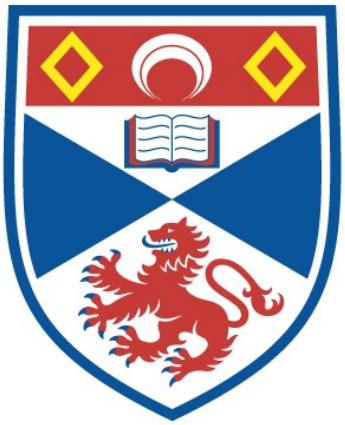
THE SUBCELLULAR LOCALIZATION, FATE AND HANDLING OF CARDIAC GLYCOSIDES BY HELA CELLS

Naji Mohammed Al-Gharably

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1986

THE SUBCELLULAR LOCALIZATION, FATE AND HANDLING OF CARDIAC GLYCOSIDES BY HeLa CELLS

A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy

By
Naji Mohammed Al-Gharably

Department of Physiology and Pharmacology,
University of St. Andrews.

Sept. 1985

Abstract

The subcellular fate of ouabain, digoxin and digitoxin after binding to HeLa cell surface was studied using a conventional ultracentrifugation technique.

Ouabain, digoxin and digitoxin codistributed with the plasma membrane marker 5'-nucleotidase immediately after binding, and cells chilled at 2° C for 24 hours retained ouabain in the plasma membrane fraction, with time these glycosides are internalized to a subcellular compartment apparently the lysosomes. Internalized ouabain showed a distribution pattern throughout the linear sucrose gradient which was similar to that of the lysosomal marker B-hexosaminidase and both activity were dependent on lysosomal integrity by showing similar response to shearing force. Internalized digoxin although showed a peak of activity in the same fraction as the lysosomal marker, but its sedimentability was insensitive to shear. Both ouabain and digoxin also responded differently to chloroquine. Chloroquine slightly reduced the accumulation of ouabain by HeLa cells, but did not much alter its excretion rate. On the other hand the amount of digoxin retained by the cell depends on the lysosomal activity. Chloroquine which reduces the lysosomal enzyme activity and inhibits lysosomal degradation result in larger accumulation of digoxin by the cell.

Antitubular agents (vinblastine and nocodazol), were found to have no detectable effect on the excretion rate of ouabain, while the excretion rate of digoxin was reduced by vinblastine.

The results are consistent with a process of internalization and turnover of sodium pumps by HeLa cells through receptor mediated endocytosis. The lipophilic property of digoxin seems to account for the differences between ouabain and digoxin handling by HeLa cells.

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CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D. Of the University of St Andrews and that he is qualified to submit this thesis in application for that degree.

date 16 Sept. 1985 signature.....

DECLARATION

I Naji Al-Gharably hereby certify that this thesis which is approximately 30000 words in length has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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ACADEMIC RECORD

I was admitted as a research student under Ordinance No.12 on 1st.Oct. 1982 and as candidate for the degree of Ph.D. on 23 Feb. 1984; the higher study for which this is a record was carried out in the University of St Andrews between 1982 and 1985.

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AKNOWLEDGEMENTS

All gratitude is due to Allah the lord of the universe for he is the one who gave me the energy, the intellectual ability and the success in this life.

It is my pleasant duty to thank Professor J.F. Lamb for his help and encouragement throughout this study. I also thank the membres of staff and my fellow research students, whose comments and advice have been appreciated. In particular, I thank Mrs. P. Ogden and Dr. G. Cramb. I must olso thank Mr. I. Laurie, Mrs. C. Voy and Mrs. M. Falls for technical assistance. I also thank ministry of interior in Saudi Arabai for the financial support I have received during this study.

Finally I owe apology to my wife and my children for lack of attendance during the preparation of this thesis.

SUMMARY

1. The subcellular localization of ouabain, digoxin and digitoxin after binding to HeLa cells, and the effect of chloroquine on the handling of these cardiac glycosides by HeLa cells were studied using cell fractionation technique.

2. Using a conventional ultracentrifugation technique the cytosol-free postnuclear pellet was layered on a sucrose linear gradient and the distribution pattern of plasma membrane marker [5-Nucleotidase], lysosomal marker [B-Hexosaminidase], mitochondrial marker [Succinate dehydrogenase] and cytosolic marker [Lactate dehydrogenase] were detected. Each of the markers was found to have a distinguish peak and distribution pattern throughout the gradient.

3. B-Hexosaminidase in the pellet fraction showed 78% latency when the assay was carried in the presence and absence of 0.1% triton-x.

4. Surface bound ouabain showed a codistribution with the plasma membrane marker 5'-nucleotidase. With time ouabain showed a distribution pattern and sensitivity to shear similar to that of the lysosomal marker, B-hexosaminidase.

5. Digoxin and digitoxn immediately after binding distributed with the plasma membrane marker, but the recovered activity of internalised glycosides did not depend on lysosomal integrity.

6. HeLa cells accumlate more ouabain than digoxin, and this accumulation unlike digoxin, is not sensitive to lysosomal enzyme activity.

7. The uptake of ouabain and digoxin into subcellular content does not depend on redistribution of soluble activity after homogenization.

8. Chloroquine greatly increases the accumulation of digoxin but does not much alter that of ouabain. This diference in responce to chloroquine is thought to be due to difference in lipophilic properties of the two glycosides.

9. Chloroquine greatly inhibits the digestion of phospholipids.

10. Chloroquine greatly inhibits the intracellular activity of B-hexosaminidase but does not affect its efflux.

11. Ammonium chloride has the same effect as chloroquine on oubain handling by HeLa cells.

12. Cells grown in suspension released ouabain very quickly and showed lack in lysosomal enzyme activity.

13. The internalization process of ouabain is energy dependent, and dissociation has a limited role in the excretion mechanism of ouabain.

14. Nocodazole and vinblastine have no detectable effect on ouabain rate of release but vinblastine reduced that of digoxin.

Introduction

Straub, 1910, as well as Clark, 1937,(as cited by Dutta,1981), were the first to show that the effect of cardiac glycosides on the heart is related to their location on the cardiac cells. Straub showed that frog heart contained special receptive sites which bound high concentration of digitalis from the perfusate. Clark calculated that bound ouabain per cardiac cell occupy very small area of the cell surface. Later on several workers, using radioactive measurement, began to explain the uptake and binding kinetics of cardiac glycosides by cardiac cell components.

Two approaches were used, the purpose of the first was to identify the specific binding site or sites for cardiac glycosides. Following this approach Schatzmann, 1953, reported for the first time that the active transport of sodium in red blood cells was blocked by cardiac glycosides. Since then, this observation has been confirmed by several authors for a wide variety of tissues, and now it is considered that a physiological function which is blocked by ouabain is associated with the sodium pump. Baker and Willis, (1972) ; and Boardman, Lamb and McCall, (1972), showed that ouabain applied to various human cultured cells,

binds to their sodium pumps and causes a decrease in potassium influx and a rise in internal sodium. Experimentally, cardiac glycosides inhibits the active transport of sodium and potassium across erythrocyte membrane only when present on the outside (Hoffmann, 1966; Perrone and Blostein, 1973), and the same applied to squid giant axon (Baker, and Willis, 1972), or cardiac cells (Okarma ,Tramell, and Kalman, 1972). Baker and Willis, (1972), showed that radioactive ouabain binds to the membranes of cultured cells in two ways, a non-specific binding which increases as the ouabain concentration increases, despite the external potassium concentration. The second was a specific binding which greatly dependent on $[K]_o$, and that specific binding occur to the sodium pumps. Ederman, (1977), and (1981), differentiated between specific and non-specific binding of ouabain in isolated human cardiac cell membranes and found that 97% of total ouabain binding occurred specifically. Lamb and Ogden, 1982a, found that 10% of the total ouabain binding occur in HeLa cells in the presence of 15mM potassium, which was considered as non-specific binding, but when digoxin or digitoxin were used a very large non-specific binding occur, but it was readily washed out, whereas the specific binding (which occur in 0K Krebs), was not (Griffiths, Lamb and Ogden, 1983). In experiments designed to analyse the factors controlling these process as well as to localize and

characterize the binding sites, the concentration of cardiac glycosides used were between 1X1E-08 M and 1X1E-05 M. Those studies have shown the existence of two classes of ouabain binding sites which have been documented in dog heart (Wellsmith, and Lindemayer, 1980), rat (Ederman, Philipp and Scholz, 1980), and human hearts (De Pover, and Goldfraind, 1979). Recently Finet, Noel and Goldfraind, (1982), have provided and indication that both classes of sites in rat heart could be Sodium-Potassium-ATPase isozymes.

In the second approach the purpose was to localize the accumulated cardiac glycosides. Sjoerdsma and Fischer, 1951 were the first to demonstrate that isolated heart bound digitoxin greatly showing 3-9 fold increase in the concentration of the cardiac glycoside found in the tissue compared to that in the medium. In, 1955, Harvey and Pieper were the first to determined the subcellular localization of ^{14}C-digitoxin accumulated by guinea-pig heart and noted that the highest digitoxin concentration was found in the post mitochondrial aqueous fraction. Dutta, Goswami, Lindower, and Marks, (1967), found that the concentration of tritiated digoxin rose in the nuclear, mitochondrial and microsomal fractions, but the fastest rise and the greatest eventual concentrations of radioactive digoxin was found in the microsomal fraction prepared by successive centrifugation of perfused guinea-pig and rat hearts homogenates. Boardman et

al., 1972, showed that growing Girardi and HeLa cells for several days in low concentration of ouabain leads to cellular accumulation of ouabain to 30-100 times that of the growth medium, and that this accumulation is sensitive to potassium concentration in the medium. In the late 1970s, several authors suggested the relation between specific-binding of ouabain and its subsequent uptake and accumulation by HeLa cells. Cook and Brake, (1978); and Lamb, and Ogden, (1982a), have shown that pumping ability of sodium pumps blocked with ouabain or digoxin recover very quickly and the rate of this recovery is similar for both glycosides. But ouabain is excreted from the cell very slowly and much slower than the excretion of digoxin (Griffiths, et al., 1983). Lamb, and Ogden, (1982a), suggested that the recovery of pumping ability of sodium pumps blocked with ouabain could be a result of pump renewal or turnover which involves the insertion into and removal of Na,K-ATPase from the plasma membrane (Karin and Cook, 1983), and that this process would be responsible for the accumulation of cardiac glycosides in HeLa cells and other tissues (Dutta, 1981).

Pollack, Tate, and Cook, 1981, showed that radioactive ouabain was internalized by HeLa cells following complete blocking of sodium pumps using 2X1E-07 M ouabain and accumulated in the lysosomal compartement (Cook, Tate and

Shafer, 1982). Griffiths, Lamb and Ogden, (1983), showed that when HeLa cells were loaded with cardiac glycosides most of the accumulation results following binding to the sodium pump and not by diffusion, and suggested an internalization model which explain that the process of internalization of cardiac glycosides bound to the sodium pump has similarities to receptor mediated endocytosis ,in which macromolecules can get access to the cell interior. This process involves several subcellular vesicles, lysosomes is believed to be one of these vesicles which has a significant role in this process.

Human cells, particularly cardiac cells, have a high sensitivity to cardiac glycosides. Several in vivo and in vitro systems were used for the study of the effect of cardiac glycosides. As described above it is clear that HeLa cells have been one of the most important systems which are used for testing the various effects and handling of cardiac glycosides. HeLa cells are used in this respect because of their nature as a human cell type which can be cloned (Puck, Marcus and Cieiura, 1956), and grown as a homogeneous population, in which physiological responses can be estimated, and recovery of physiological changes can be observed. The second major reason for using HeLa cells is because these cells have retained the high sensitivity of human cells to cardiac glycosides, and provides a good model

for the uptake and excretion of cardiac glycosides by human heart. This does not apply for other human cell types such as human erythrocytes (Lamb and Ogden, 1982a).

Although ouabain, digoxin, and digitoxin are structurally and pharmacologically similar (Goodman and Gilman, 1970)), yet they have shown some differences in the accumulation and release of these cardiac glycosides from HeLa cells (Griffiths et al. 1983). But as expected they behaved similarly in binding to sodium pumps and in the recovery of the pumping ability. In the present work the subcellular localization of these cardiac glycosides after acute and chronic treatment of HeLa cells were studied using an ultracentrifugation technique, to provide evidence for the idea that cardiac glycosides are internalized to the lysosomes through receptor-mediated endocytosis. Additional aims are to try to explain the possible reasons for accumulation and release differences between these glycosides using chloroquine, and some other conditions, as an interfering factors in the handling process of these cardiac glycosides by HeLa cells.

Receptor mediated endocytosis:-

This is a complex process whereby receptors on the cell surface bind specifically an extracellular macromolecule and deliver it to the lysosomes. The study of receptor mediated endocytosis has emphasized on several subcellular vacuoles termed the vacuolar system, which is the structure that participate in endocytosis. This structure consist of the plasma membrane on the cell surface , coated pits, coated vesicles and endosomes which participate in delivery of internalized molecules to the secondary lysosomes which contains hydrolases enzymes responsible for the degradation of the ligand (Goldestine, Anderson and Brown, 1979; and Stahl and Schlesinger, 1980). In many cases rapid internalization of receptor-bound proteins is achieved by the clustering of receptors in specific parts of the cell surface membrane called coated pits that bud into the cell during endocytosis to form coated vesicles (Anderson, Brown and Goldstein, 1977; and Brown, Anderson, and Goldstein, 1978). These vesicles then move to perinuclear regions and fuse with lysosomes (Silverstein, Steinman and Cohn, 1977). There is some evidence that coated pits and coated vesicles are part of the subcellular organelles because they have a characteristic structure and protein composition and they can be separated (Pears, 1975 and 1976). Pears also observed that the coat of coated vesicles isolated from

variety of animal cells is composed of a single protein called Clathrin. It is suggested that the coated vesicles lose their Clathrin coat and the Clathrin moves back to the cell surface (Goldstein, et al., 1979; and Helenius, Marsh and White, 1980), and the ligand receptor complex is delivered to endosomes (Pastan and Willingham, 1981). These endosomes are prelysosomal heterogeneous acidic endocytic vacuoles through which internalized ligands pass into the lysosomes. Although it is still difficult to isolate endosomes, they can be distinguished from lysosomes by brief exposure to some endocytosed marker such as horseradish peroxidase. Endosomes have been described in a variety of cell types and receptor mediated endocytosis systems and are involved in the transport of internalized ligands into lysosomes (Helenius, Mellman, Wall and Hubbard, 1983). The pathway followed by endocytic vacuoles varies with the cell type, membrane fusion occurs at many levels in the endocytic pathway between two protoplasmic faces (Steinman, Mellman Muller, and Cohn, 1983). The fusion of internalized vacuoles with lysosomes can be studied by following the appearance of internalized contents in acid hydrolase-positive vacuoles. The development of a low pH in an endocytic vacuole can be visualised with acridine orange (Hart and Young, 1975). Kielion and Cohn, (1980), used this technique to follow the fusion of endocytosed yeast particles in Macrophages. The macrophagic studies have

indicated that ligand and receptor enter the cell together (Wall and Hubbard, 1981).

Receptor-mediated endocytosis has been proposed as the mechanism of internalization of many ligands. Peptide hormones (e.g Insulin, epidermal growth factor; Schlesinger, Shechter, Willingham and Pastan, 1978), low density lipoprotein (Goldstein, Anderson and Brown, 1979), and lysosomal enzymes (Sando, Titus-Dillon, Hall, and Neufeld, 1979), are some of the examples of ligands internalized by receptor-mediated endocytosis. Most high affinity binding sites which mediate the endocytosis of specific ligands deliver ligands to lysosomes. In such system the lysosomes play a significant role. It is believed that in the lysosomal stage, ligand split from the receptor. Goldstein et al., (1979), showed that low density lipo protein (LDL) when internalized is split into cholesterol subunits. Each (LDL) particle has a core of some 1,500 cholesterol molecules chemically bound to fatty acid chain. (LDL) particles bind to specific cell surface receptor and then internalized in membrane bound vesicles and transported to the lysosomes. The lysosomal hydrolases, break down the (LDL) particles, degrading the binding protein, and splitting the fatty acid from the cholesterol molecules. Helenius, Kartenbeck, Simons, and Fries, (1980), showed that the Semliki Forest Virus which enters BHK-21

cells after attaching to the cell surface was rapidly trapped into coated pits, internalized by endocytosis in coated vesicles, and sequestered into intracellular vacuoles and then into the lysosomes where viruses lost their coats.

It is also believed that after the ligand split from the receptor, the receptor is recycled and used many times (Schwartz, Fridovich and Lodish, 1982). Which indicates that binding receptors can deliver ligands to lysosomes but the receptor is not degraded. Steinman et al. (1983) carried out a kinetic study in which they compared the molecules per cell bound to plasma membrane with molecules per cell internalized per hour, in several systems where receptor mediated endocytosis takes place. They found that during a continuous exposure to ligand, cells internalized 5-30 times the maximum number of surface-bound molecules.

The process of receptor-mediated endocytosis can be inhibited by low temperature (Steinman, Silver and Cohn, 1974; and Carpenter and Cohen, 1976). The uptake of an endocytic tracer like horseradish peroxidase (HRP) is undetectable at 4°C by both quantitative or cytochemical assays (Steinman and Cohn, 1972; and Steinman et al., 1974). Similarly internalization of bound ligands does not occur in chilled cells. Iacopetta and Morgan, (1983), showed that endocytosis of diferric transferrin and accumulation of its iron by isolated rabbit reticulocytes

was inhibited at an incubation temperature of 4 C. Wall and Hubbard, (1981), described that beside low temperature, brief formaldehyde fixation of rat hepatocyte from isolated perfused liver leads to the block of internalization of iodine labelled ligands from the perfusate.

Separation of lysosomes:-

Lysosomes are membrane bound vacuoles rich in acid hydrolases. There are two types of lysosomes , a primary lysosome is the vacuole which contains hydrolases, and a secondary lysosome which is a vacuole rich in acid hydrolases that has acquired substrates.

Cell fractionation has been a classical procedure for isolation of lysosomes. Homogenization of cells or tissue must be performed as gently as possible in order to minimize physical damage to lysosomes. In most cases homogenizers with a rotating pestle as first devised by Potter and Elvehjem (1936), or the homogenizer of Dounce, Witter, monty, Pate and Cottone (1955), provide enough shearing force to break cells without too much damage to the subcellular particles. Much gentler means, such as pipetting the suspension of cells several times, are used to disrupt polymorphonuclear leukocytes from rabbit peritoneal exudates (Cohn and Hirsch, 1960). Other means of cell homogenization are also used (Beaufay, 1972).

Methods of tissue homogenization can be evaluated by morphological and biochemical criteria. Phase contrast microscopy easily shows the degree of cellular disruption achieved and detects aggregation artefacts. It is also easy to examine gross alteration of subcellular organelles by this mean. Biochemical estimation allow more quantitative evaluation of lysosomal structure integrity. The lysosomal membrane integrity is essential for lysosomal hydrolases latency and sedimentability. For instance the sedimentability and enzyme activity latency of catalase can be used as an indication for the preservation of liver or kidney peroxisomes (Baudhuin, Muller and Poole, 1965b), also the sedimentability of B-Hexosaminidase can be used to detect the effect of shearing force on HeLa cells lysosomal integrity (Cook et al., 1982). Other subcellular organelles integrity such as mitochondria can be evaluated similarly (Bendall and De Duve, 1960).

Centrifugation is still the most important physical method which has been successfully used to isolate lysosomes from various tissue homogenates. Two methods are used for this, differential centrifugation methods, which separate subcellular particles sedimenting at different velocities, and isopycnic methods, which separate subcellular particles equilibrating at different densities. Other methods have been used for separation of subcellular particles, such as

electrophoresis (Davenpoort, 1964; and Harms, Kern, and Schneider, 1980).

Purified lysosomes have been separated because the density of most lysosomes is high enough to distinguish them from other subcellular particles. But considerable purification has also been obtained by modifying lysosomal density using latex (Wetzei and Korn, 1969), or Triton-WR-1339 (Wattiaux, et al., 1963), or Dextran-500 (Beaufay, 1972). Although these substances succeeded in producing pure lysosomes, they may induce an increase in lysosomal size which may render them more susceptible to the shearing force produced during homogenization. Indication of an increased lysosomal fragility resulting from an increased size have been found in Triton-WR-1339 loaded lysosomes (Wattiaux, Wibo and Bandhim, 1963).

Sucrose is the solute commonly used to provide sufficient osmotic potential in the suspending medium of separated lysosomes. Sucrose sometimes replaced by mannitol if it interferes with the assay of enzymes. However this compound does not always ensure a maximum preservation of lysosomes (Bowers, Finkenstaedt and De Duve, 1967). Saline solutions are usually avoided because they agglutinate the subcellular organelles in homogenates of liver (De Duve and Berthet, 1954), and of polymorphonuclear leucocytes (Cohn and Hirsch, 1960). However 0.2 M potassium chloride

solution is used to prevent agglutination in spleen homogenates (Bowers et al., 1967). Small amounts of EDTA is the substance most widely used to prevent agglutination of subcellular organelles and seems to have no undesirable effect (De Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955).

It is well known that many of the lysosomal enzymes are most active at an acidic pH. Mego (1971), has shown that the intralysosomal pH is near to 5. It is because of this pH that lysosomes accumulate weak bases (Wibo and Poole, 1974) such as chloroquine, ammonium chloride, and amantadine. These agents elevate the lysosomal pH as much as 1.7 pH units (Ohkuma and Poole, 1981), and when these agents are removed from the medium, the pH rapidly returns to values close to the original. This rise in intralysosomal pH could alter the activity of some of the lysosomal enzymes, and thereby impair lysosomal digestion.

The effect of chloroquine:-

Many studies have documented the capacity of chloroquine, and other weak bases, to influence lysosomes both in vivo and in vitro.

Abraham, Golberg and Grosso,(1967), have found that the lysosomes of heart became enlarged and abnormal following chloroquine treatment. Several authors have reported the capacity of chloroquine to stabilize the membranes of rat liver lysosomes in vitro (Miller and Smith, 1966; and Ignarro, 1971a), and retarded the release of hydrolytic enzymes from rabbit liver granules induced by several substances, such as progesterone (Weissmann, 1964). Alison and Young, (1964), have found that chloroquine is selectively taken up and concentrated in lysosomes of cultured cells, and retarded lysosomal damage.

On the other hand, evidence has appeared to show that both chronic and acute treatments of rat cytoplasmic granules and isolated rat liver slices with chloroquine induced moderate to marked lysosomal acid hydrolases alterations leading to an increased free enzyme activities; this indicates that chloroquine induces lysosomal membrane labilization (Filkins, 1969; Malbica and Hart, 1971; and Fedorko, Hirsch and Cohn, 1968a). More recently Ngaha and Akanji, (1981), showed that chronic administration of chloroquine to rats results in increased urinary excretion of lysosomal acid phosphatase. In vitro studies of rat lysosomal suspension in the presence of chloroquine resulted in a marked lysosomal acid phosphatase release which was antagonized by addition of acetylsalicylic acid, a lysosomal

stabilizer (Ignarro and Colombo, 1972). Jessup et al.,1982 have found similar effect of ammonium chloride on mouse peritoneal macrophages and human peritoneal blood monocytes.

The capacity of chloroquine to influence intralysosomal pH could alter the activity of some of the lysosomal enzymes, and thereby impair lysosomal digestion which lead to the accumulation of undigested materials. Matsuzawa and Hostetler, (1979), studied the effect of chloroquine on rat liver lysosomal phospholipases A and C, and showed that chloroquine is a potent inhibitor of these lysosomal enzymes. Essentially all of the accumulated phospholipids in rat liver were found in the lysosomes (Matsuzawa and Hostetler, 1980). They also suggested that chloroquine induced lipidoses results from a block in intralysosomal phospholipids catabolism and not due to an increase in phospholipid synthesis and that the inhibition mechanism of phospholipases caused by chloroquine involves a substantial degree of accumulation of chloroquine in cell lysosomes (Hostetler and Richman, 1982).

There is a considerable evidence that beside lysosomes, other parts of the vacuolar system are affected by chloroquine. Fedorko, (1967) and (1968), observed abnormalities in the vacuolar system of polymorphs and lymphocytes from patients given chloroquine, and in leukocytes and pancreatic cells of rats treated with the

drug. Further evidence that chloroquine affect the vacuolar system was obtained by Macomber, Sprinz and Tousimis, (1967), and Warhurst and Hockley, (1967), who observed malarial parasites (plasmodium berghei) treated with chloroquine. The parasites, living within erythrocytes, apparently endocytose haèmoglubin in cytoplasmic vacuoles. Following chloroquine treatment, the phagocytic vacuoles enlarged, myelin figures appeared, and cell death followed. Weissmann, (1969), suggested that the parasites filled with phagosomes which could not receive hydrolases from primary lysosomes, became starved of nutrient and died.

As chloroquine affect the vacuolar system it is expected that chloroquine, and other weak bases, interfere with receptor- mediated endocytosis. Sando, Titus-Dillon, Hall and Neufeld, (1979), showed that chloroquine and ammonium chloride interfere with receptor- mediated uptake of lysosomal enzymes. They suggested that chloroquine inhibited the internalization step of the process. Similar result was obtained by Fitzgerald, Morros, and Saelinger, (1980). They described that chloroquine blocked the clustering and internalization of pseudomonas exotoxin by mouse fibroblasts. The previous studies suggested that chloroquine interferes with the internalization step of receptor mediated endocytosis, but on the other hand several studies suggested that chloroquine affect the lysosomal step

of the process. Helenius, Kanterbeck, Simons, and Fries, (1980), showed that two possible mechanisms in which chloroquine interferes with the lysosomal stage of receptor mediated endocytosis of Semliki Forest virus by BHK-21 cells, the first was that chloroquine may inhibit a lysosomal hydrolase required for penetration of the virus into lysosomal compartment, or inhibition could be caused by the elevation in lysosomal pH.

Part I

The subcellular localization of ouabain, digoxin and digitoxin after binding to HeLa cell surface

Methods

Cell culture:-

HeLa S3 cells used in this work were supplied as monolayer cultures, kept in suspension of growth media containing 10% v/v dimethyl sulphoxide (DMSO), frozen to -80 C, and stored in liquid nitrogen (union carbide LR-40, container).

Cells were maintained in the laboratories by rapidly thawing the frozen cell stock in Basal Medium Eagle's and diluting to appropriate cell number. The cell suspension of 1X1E-06 cell/ml was transferred into Roux bottle with a growing surface area of 120 cm^2. containing 100 ml of basal medium eagle's supplemented with 10% v/v newborn calf serum , L-glutamine (final concentration 0.281 mg/ml), and penicillin-streptomycin (100 units/ml). The cultures were gassed with a mixture of 5%CO2 and 95% air, tightly sealed, and grown to confluence (4 days) at 37°C. When cells were needed for experimental purposes, the media was decanted and the cells were rinsed with 20 ml of Earle's balanced salts (magnesium and calcium free) solution, and 5ml of 0.25% trypsin solution was added to the culture and left at 37°C for 20 min. When the cells had been detached the trypsin was neutralized by adding 20 ml of growth media. Cells were then dispersed by passing the cell suspension through a sterile stainless steel needle (1.1mm internal diameter),

using sterile 10 ml plastic syringe. One ml of this cell suspension was taken for cell number and cell volume estimation and the appropriate cell concentration was diluted in the growth media, transferred to Roux bottle with a growing surface area of 120 cm^2., and seeded with 5X1E-06 cells in 100ml of growth media. The cultures were gassed with a mixture of 5% CO and 95% air, tightly sealed with tape, and incubated at 37°C. All culturing procedures were carried under aseptic condition in a "Gelman" laminar flow cabinet.

Cell number and volume estimation :-

The number and volume of trypsinized cells diluted in suspension was measured using a coulter counter (ZF)connected to a channalyser (C 1000). One ml of cell suspension was added to 19 ml of "isoton" counting fluid in coulter vials. The coulter counter measures a fixed volume of 0.5 ml through an internal diameter of 100 µm. Trypsin does not alter the cell volume (Lamb and Mackinnon, 1971).

Assays:-

Proteins :- was assayed according to Lowry et al (1951), with the addition that samples containing sucrose were treated with 10% w/v trichloroacetic acid to precipitate protein as follows: 0.7 ml 10% TCA was added to 0.5ml sample, left in ice for 30 min, centrifuged at 2400

rpm. for 15 min in (MSE) coolspin centrifuge, washed with 1 ml of 70% alcohol, centrifuged again at 2400 rpm. for 15 min, tubes were converted and dried at 37°C for 30 min, and protein was determined according to Lowry et al., (1951). A standard curve of bovine serum albumin for quantities up to 400 µg/1.2 ml was obtained at 750 nm using "Pye unicam" sp6-550 uv/vis spectrophotometer. The curve was linear over the range.

Enzymes assays:-

B-hexosaminidase:- was assayed according to Barrett (1972). The samples were incubated for 30 min, in the presence of p-nitrophenyl-N-acetyl -B-glucosaminide as substrate at pH 4.6, and 0.3 M NaCl was added to increase the activity, and 1.5 ml of 0.5 M sodium bicarbonate - sodium carbonate buffer (pH=10) was used as stopper. The free aglycone was determined by comparing the extinction measured in the assay solution with standard curve of p-nitrophenol solution (0.04 - 0.4 µ mol./3 ml), measured at 420 nm

5'-nucleotidase :- (EC 3.1.3.5), was assayed as described by Cramb and Dow (1983). When the enzyme distribution pattern throughout the sucrose gradient was detected 3 volumes of 50 mM tris base buffer (pH 7.4), containing 2 mM calcium chloride was added to each gradient

fraction sample, since the sucrose gradient buffer contains 1 mM EDTA which inhibits 5'-nucleotidase (Brake et al.,1978).

Lactate dehydrogenase :- (EC 1.1.1.27), was assayed as described by Bergmeyer and Brent (1974), using B-nicotinamide adenine dinucleotide, reduced form as substrate. The change in extinction at 340 nm was measured and the volume activity was calculated from the mean extinction change measured at 1, 2, 3, and 4 min,

Succinate dehydrogenase :- (EC 1.3.99.1) was assayed according to Pennington (1961). 0.25 ml of sample was added to 0.75 ml assay solution containing 0.1% INT. with potassium phosphate buffer pH 7.4 in duplicate (15 ml) glass stoppered "Quikfit" tubes. The produced Formazan was extracted with 4ml ethyl acetate and the extinction measured at 490 nm was compared with standard solution of Formazan in ethyl acetate (2.5 - 15 µg/4ml).

Cell fractionation :-

cells were fractioned in the conventional way to obtain a particulate fraction containing mainly plasma membrane , lysosomes, and mitochondria. Four Roux bottles were used in each experiment, the media was decanted and cells were washed with 20 ml of serum - free media (ice cold), and cells were collected by scraping off the glass surface using

a small brush, then resuspended in 10 ml of o.25 M sucrose - 10 mM tris - 1 mM EDTA (homogenizing buffer, pH = 7.2-7.4). The cell suspension was then transferred to a Dounce homogenizer (Wheaton) and 40 strokes of the tight pestle "B" (0.0025" - 0.0055" clearance) were applied, and the resulting homogenate was centrifuged at 1100-g for 1 min, in (MSE) coolspin to remove the intact cells and nuclei. The cell-free lysate (F-I), which is the supernatant of the last step was recentrifuged at 22,000 g. for 20 min, in a 5 ml ultra clear centrifuge tubes using SW65 rotor in (Beckman L2-65B), ultracentrifuge at 2^{o}C to produce the particulate fraction (P-II) which pellets in the bottom of the tube and the cytosolic fraction (S-III), which is the supernatant, this was decanted and the inside wall of the tube was wiped with a kim - wipe. The particulate fraction was resuspended in 3 ml homogenizing buffer which contains 1mM EDTA to prevent agglomeration. This suspension was then layered on (28 ml) linear 20%-55% w/w sucrose gradient on the top of 3 ml 65% w/w sucrose cushion in "ultra clear" centrifuge tube with a capacity of 38.5 ml, the sucrose gradient was cool down before layering the particulate fraction to 2^{o} C The gradient was then centrifuged at 25,000-g using SW27 rotor in (Beckman L2-65B) ultracentrifuge for 2 hours at 2^{o} C. After centrifugation was completed the gradient was fractioned to 17 fractions each fraction is 2 ml using long stainless steel needle inserted very slowly to the bottom of

the gradient and connected to a Watson-Marlow pump (type MHRG 200), with controlled flow. The fractionation starts from the bottom of the gradient that is to say fraction number 1 resembles the cushion while fraction number 17 resembles the overlay. A schematic summary is shown in (fig.1.1.).

Determination of the distribution pattern throughout the linear sucrose gradient:-

The distribution pattern of subcellular enzymes markers was determined by measuring the activity of these enzymes in each of the 17 sucrose gradient fractions, and the distribution pattern of ouabain, digoxin, and digitoxin was determined by mixing 0.5 ml of each gradient fraction with 5 ml of scintillation fluid for radioisotope determination in a "Packard Tri Carb" scintillation spectrophotometer (model 3255).

Preparation of the linear sucrose gradient :-

Using a U shape tube, a linear 20-55% w/w sucrose gradient (28 ml), on a 3 ml 65% w/w sucrose cushion was prepared as outlined in (fig.1.2).

FIG. 1.1: CELL FRACTIONATION

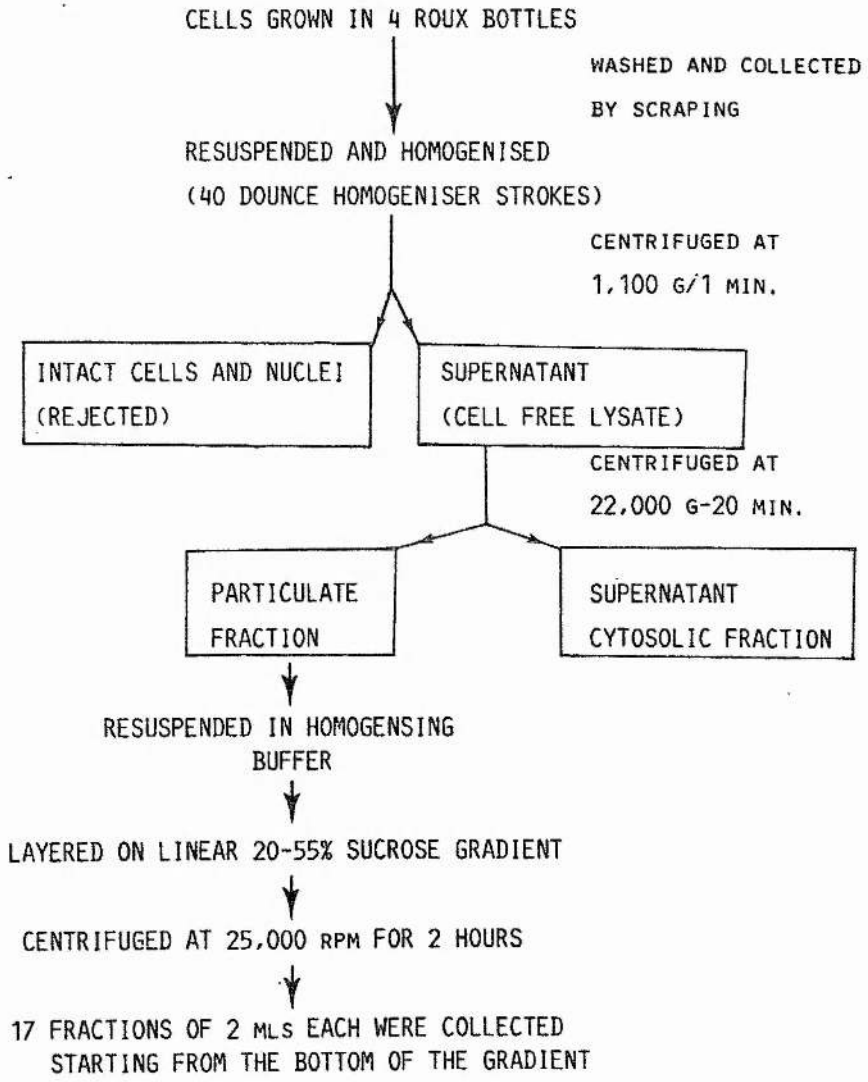


Fig.1.2: Preparation of the linear sucrose gradient.

The linear 20-55% w/w sucrose gradient was prepared using a U shape tube with two taps, tap "A" connecting the two sides of the tube (side 1 and 2), and tap "B" delivering the gradient mixture to the centrifuge tube. Side "1" of the tube contains a magnetic stirrer and before preparing the gradient the tube set was placed on a stage magnetic stirrer. 14 ml of 55% w/w sucrose solution containing (10mM tris - 1mM EDTA), was pipetted into side "1", with tap "A" and "B" are closed. Tap "A" was opened till the solution reach the bottom of side "2" , and tap "A" was closed. 14 ml of 20% w/w sucrose solution containing (10mM tris - 1mm EDTA), was added to side "2", and tap "A" was completely opened. When the level of the two solution settled the stirrer was put on , and tap "B" was opened slowly to allow the gradient mixture to slide down on the inside wall of the centrifuge tube and lie on the top of 3 ml of 65% w/w sucrose solution cushion. A reproducible linear sucrose gradient was obtained with density distribution as described in (table 1.a), measured by 0 - 50% sucrose refractometer.

Fig.1.2

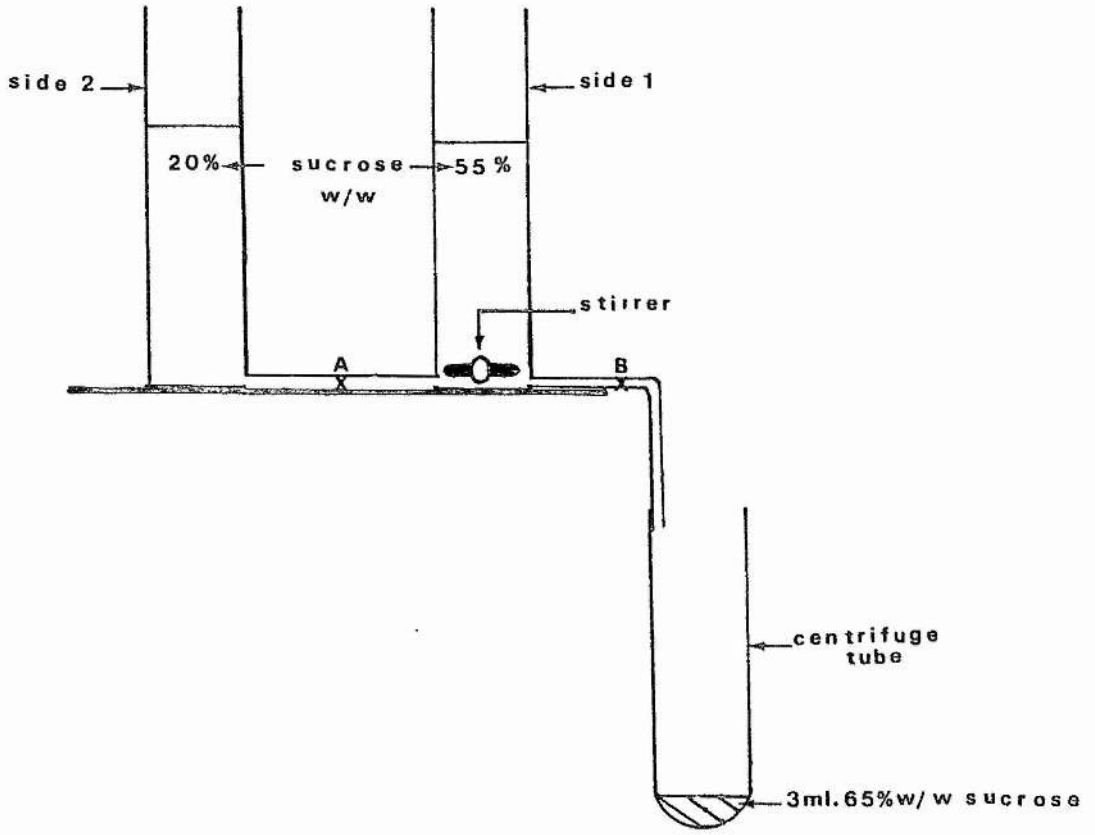

side 2
side 1
20%
sucrose
w/w
55 %
stirrer
A
B
centrifuge
tube
3ml. 65%w/w sucrose

Table 1.a

The linear sucrose gradient density distribution

fraction number	sucrose % W/W
1	65%
2	>50%
3	>50%
4	>50%
5	48-50%
6	45-46%
7	42-43%
8	39-40%
9	37-38%
10	35-36%
11	33-34%
12	30-31%
13	28-29%
14	25-27%
15	22-24%
16	16-18%
17	overlay 10%

Cardiac glycoside treatment :-

Chronic treatment :-

Cells were grown in Roux bottles as described under tissue culture for 48 hours in the presence of 1X1E-08 M cardiac glycoside with 0.5 uci/ml [3H] radioactive ouabain or digoxin. Media was decanted and cells were rinsed with 30 ml of ice cold 5mM K^+ Krebs containing 1% v/v newborn calf serum (table 1.b), then collected by scraping off the glass surface using a small brush, and suspended in homogenizing buffer. 0.1 ml sample was taken and mixed with 5 ml scintillation fluid for radioactive measurement and bound activity was expressed as n mol/mg protein.

Cardiac glycoside pulsing :-

Cells were grown to confluency in Roux bottles, the media was decanted, and cells were washed with warm 0mM K Krebs solution. 25 ml of K free Krebs supplemented with 1% v/v dialysed newborn calf serum (table 1.b), containing 2 X1E-07 M cardiac glycoside and 1uci/ml [3H] radioactive ouabain or 0.5 uci/ml [3H] radioactive digoxin and digitoxin was added to the culture for 20 min, at 37° C. Cells were then washed with 30 ml of 5 mM k^+ Krebs at 37°C for 15 min, to wash off the non specific binding (Griffiths et al., 1983), then returned to growth medium.

(table 1.b)

composition of 5mM K Krebs solution:

	mM
NaCl	137
KCl	5.4
$CaCl_2$	2.8
$MgSO_4.7H_2O$	1.2
NaH_2PO_4	0.3
KH_2PO_4	0.3
HCl	12
Trisma base	14
Glucose	0.2%w/v
Calf serum	1.0%v/v

PH=7.4

K free Krebs was prepared by omitting KCl, replacing KH_2PO_4 by NaH_2PO_4, and replacing calf serum by dialysed calf serum.

The cells were left for 9 hours (digoxin and digitoxin), or 24 hours (ouabain) ,then the incubation media was decanted and cells were washed with 30 ml of ice cold 5 mM K Krebs and collected as described above. Samples were taken at times between 0-9 hours (digoxin and digitoxin) or 0-24 hours (ouabain). The bound activity was measured and expressed as described under chronic treatment.

The effect of homogenization on the release and sedimentation of ouabain and digoxin:-

Cell were grown to confluency on Roux bottles, loaded with [3H] ouabain, and returned to normal growth medium for 24 hours. The cell suspension was then severely homogenized using 100 Dounce homogenizer strokes. The cell free lysate (F-I) was prepared as described before and then layered on the linear sucrose gradient. The activity of both B-hexosaminidase and ouabain throughout the gradient were detected as described before.

The same experiment was repeated on cells loaded with [3H] digoxin which were returned to normal growth medium for 10 hours. Another homogenizing technique was applied to cells loaded with [3H] digoxin. This technique (shear sensitivity analysis) was carried out as described by Cook, et at, (1982). HeLa cells were grown to confluency on Roux bottles, pulsed with [3H] digoxin, then returned to fresh

media for 10 hours. The cells were then washed and resuspended in 15 ml buffer and homogenized with the tight pestle "B", using an increasing number of strokes (10, 20, 40, 60, 80, and 100 strokes). The microscopic examination showed that complete lysis was achieved with 100 strokes. A sample was removed after each number of strokes, and the total B-hexosaminidase and [3H] digoxin activities were determined. Remaining samples were then centrifuged at 1100g for 1 min, in (MSE) coolspin centrifuge to produce the cell-free lysate (F-I) which was analysed for both B-hexosaminidase and [3H] digoxin activity. The remaining cell-free lysate samples were further centrifuged at 22,000g for 20 min, in SW65 rotor as described under cell fractionation and the resulting particulate fractions (P-II) was resuspended in homogenizing buffer and assayed for B-hexosaminidase and [3H] digoxin activities.

Dextran treated cells :- In order to obtain a dextran filled lysosomes, HeLa cells were grown in Roux bottles for 48 hours at 37 C in 100 ml (BME) containing 2% of dextran-500, supplemented with 10% v/v newborn calf serum, 100 units/ml penicillin-streptomycin, and 0.281mg/ml L-glutamine, then proceed as described under cell culture. This experiment was based on the method described by Beaufay (1972), in which dextran filled lysosomes were separated from rat liver injected with Dextran-500.

Materials

HeLa cells S3 are supplied as monolayer cultures by Flow laboratories ltd. Basal Medium Eagle's (BME) with Earle's salts (table 1.c), and newborn calf serum were obtained either from Flow laboratories or Gibco limited. L-Glutamine, penicillin- streptomycin, and trypsin solution were obtained from Gibco limited. Bovine serum albumin, B-nicotinamide adenine dinucleotide reduced form (B-NADH, disodium salt, grade III), digoxin, digitoxin, Ouabain, Folin and Ciocaltue's phenol reagent (2 Normal), adenosine-5'-monophosphate(AMP sodium salt) ,iodonitrotetrazolium (Formazan), p-iodonitrotetrazolium violet (grade I), p-nitrophenol, p-nitrophenyl-N-acetyl-B-D-glucosaminide (crystalline), Trisma base, and triton X- 100 (octyl phenoxy polyethoxyethanol) were obtained from Sigma chemical company. Dextran (T500) was obtained from pharmacia fine chemical. [3H] adenosine 5'-monophosphate (3H-AMP, ammonium salt), [3H] digoxin, and [3H] ouabain were obtained from Amersham international plc (Amersham U.K.). [3H] digitoxin was obtained from New England nuclear. Scintillation fluid was a mixture of toluene, triton X- 100, 2,5-diphenyloxazol, and 1,4-di[2- (5- phenyloxazolyl)] - benzene, obtained from BDH chemical ltd. (Poole, England). Other chemicals were of Analar grade from BDH chemical Ltd. (Poole, England).

(table 1.c)

Basal Medium Eagle with Earle's salts:

Ingredient	mg/liter
L-Arginine	21.06
L-Cystine disodium salt	14.51
L-Histidine HCl	10.50
L-Isoleucine	26.23
L-lysine HCl	36.53
L-Methionine	7.46
L-Phenylalanine	16.51
L-Threonine	23.82
L-Tryptophan	4.08
L-Tyrosine disodium salt	22.51
L-Valine	23.43
Biotin	1.00
D-Ca pantothenate	1.00
Cholne chloride	1.00
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavine	0.10
Thiamin Hcl	1.00
$CaCl_2 .2H_2O$	264.9
KCl	400.0
$MgSO_4 .7H_2O$	200.0
NaCl	6800.0
$NaHCO_3$	2250.0
$NaH_2PO_4 .2H_2O$	158.3
D-Glucose	1000.0
Phenol red sodium salt	17.00

Results

Subcellular, enzymes, markers are often used to identify the availability, the purity, or the distribution of certain subcellular structures within a separated subcellular fraction. That is why these markers are widely used as the best criterion to evaluate the validity of a certain subcellular separation technique.

In this work the enzymes were used not only as subcellular markers, but to identefy the destination of certain cardiac glycosides bound to HeLa cells as well.

In order to localize the glycoside during the subsequent uptake , a conventional ultracentrifugation technique was used. Cook, Tate and Shafer (1982), used this technique for the subcellular localization of Ouabain in HeLa cells which were grown and loaded with ouabain in suspension.

Subcellular enzyme markers :-

The activity of several enzymes throughout the linear sucrose gradient has been detected. 5'-Nucleotidase, a plasma membrane marker (Brake, Will and Cook, 1978), B-Hexosaminidase, a lysosomal marker (Barrett 1972), Succinate dehydrogenase, a mitochondrial inner membrane marker, and lactate dehydrogenase, a cytosolic marker, were

detected. The amount of these enzymes activities which were recovered in the particulate fraction (P-II), or remained in the cytosolic fraction (S-III), were also estimated.

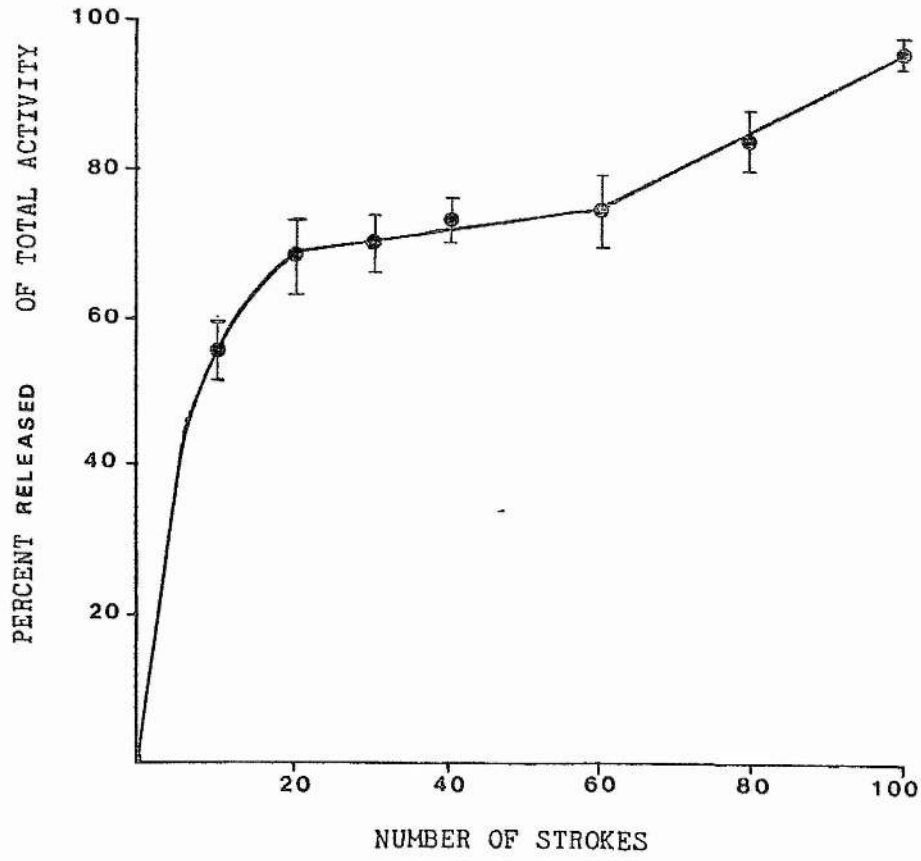
Cell fractionation:-

HeLa cells were homogenized by applying 40 Dounce homogenizer strokes in order to obtain a particulate fraction (P-II), rich with intact lysosomes. If this number was exceeded ,more B-hexosaminidase activity was released to the cell free lysate (F-I), fig.1.3.a, but less activity was recovered in the particulate fraction (fig.1.3,b). This was determined by measuring the percentage of total cell homogenate activity that sediment in the particulate fraction after a given number of homogenizing strokes. The optimum homogenizing strokes were ranging between 30-40 strokes,(fig.1.3,b). To estimate that the B-hexosaminidase activity recovered in the particulate fraction was coming from intact lysosomes, it was assayed in the presence and absence of 0.1% triton X-100. B-hexosaminidase showed 78% latency in the particulate fraction. The specific activity of the enzyme was 0.537+0.035, and 0.958+0.047 u mol/mg protein /hour, for controls and triton treated particulate fractions respectively.

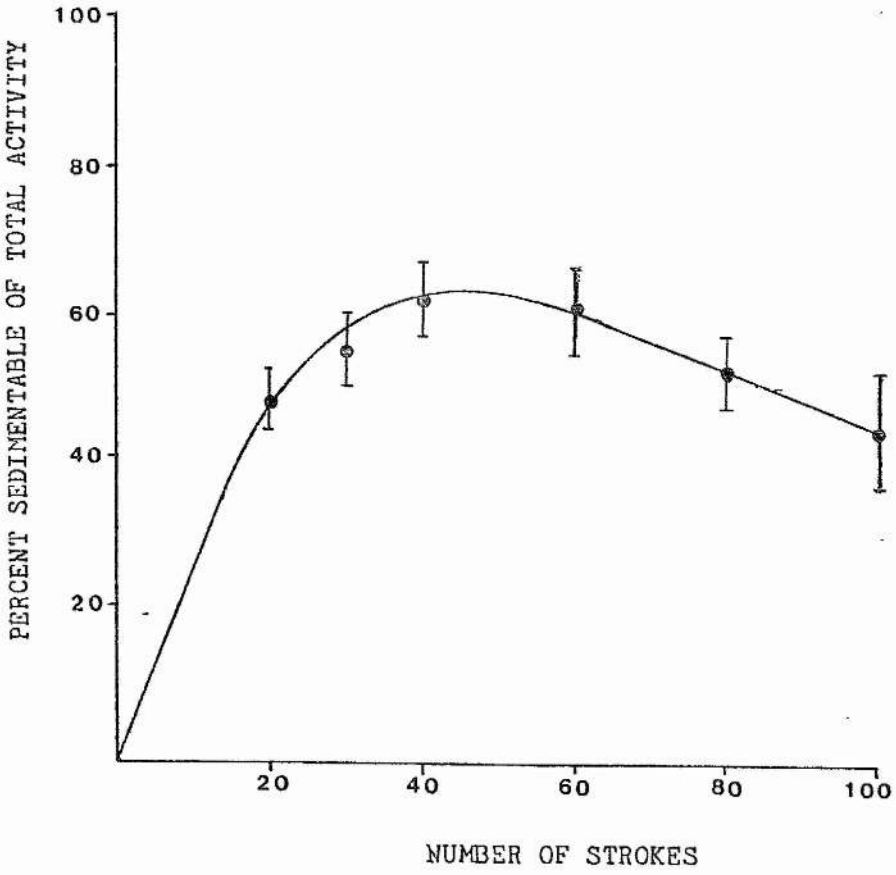
Fig.1.3,a and b : The effect of homogenization on B-hexosaminidase release and recovery.

The cell suspension was exposed to an increasing number of Dounce homogenizer strokes. The cell free lysate (F-I) was prepared at each number of strokes, and an aliquot was assayed for B-hexosaminidase activity. The result was plotted as the percent of total activity in the original cell suspension which was released into the cell free lysate as a function of the strokes applied . In (fig.1.3,a), as the homogenization increases the activity released into the cell free lysate increases too. Fig.1.3,b, shows that the amount of activity recovered in the particulate fraction (P-II) was increased as the homogenization increases to 40 strokes reaching a maximum of 62% of the total initial activity. Further homogenization lead to decline of the activity recovered The results are the mean of three experiments ±S.D.

(fig.1.3,a)


PERCENT RELEASED OF TOTAL ACTIVITY
100
80
60
40
20
20
40
60
80
100
NUMBER OF STROKES

(fig.1.3.b)


PERCENT SEDIMENTABLE OF TOTAL ACTIVITY
100
80
60
40
20
20
40
60
80
100
NUMBER OF STROKES

Amount of enzymes recovered in the particulate fraction:-

The amount of each enzyme activity of the relevant markers pelleted in the particulate fraction, or that remained in the cytosolic fraction was estimated. The activity which was recovered varied between 73% - 102% of the total cell free lysate activity.

As shown in (fig.1.4,a), about 80% of the total cell free lysate activity of 5'-nucleotidase was recovered in the particulate fraction, while 15% remained in the cytosolic fraction. Less activity of both lysosomal marker and mitochondrial marker was recovered in the particulate fraction. 73% of B-hexosaminidase activity was recovered and 26% remained in the cytosolic fraction as shown in (fig.1.4,a). While 75% of succinate dehydrogenase activity was pelleted and about 25% remained in the cytosolic fraction,(fig.1.4,b). The cytosolic marker, lactate dehydrogenase, gave a different picture as very little or no activity was recovered in the particulate fraction (3%), and nearly all the cell free lysate activity remained in the cytosolic fraction,(fig.1.4,b).

Enzymes distribution patterns:-

When the pelleted activities of the previous enzymes was layered on the linear sucrose gradient, each enzyme showed a distinguishable peak and a different distribution patterns throughout the gradient .

5'-nucleotidase, the plasma membrane marker, gave a peak of activity at 30 -31% w/w sucrose, and very little activity moved down towards the dense part of the gradient (fig.1.5).

B-hexosaminidase pelleted activity entered the gradient and peaked at 35 -36% w/w sucrose. This peak was well distinct from that of the plasma membrane marker. It also gave a different distribution pattern, the activity was broadly distributed throughout the gradient with a substantial amount of activity remained in the overlay at the top of the gradient (fig.1.6), which might represents the contamination of the particulate fraction with the cytosolic activity of this enzyme, and/or the activity released from ruptured lysosomes during the resuspending of the particulate fraction.

All the succinate dehydrogenase activity entered the the gradient and peaked at a much denser part of the gradient (39 - 40% w/w sucrose), and distributed away from the overlay giving a limited distribution pattern in only 8 fractions. (fig.1.7).

Lactate dehydrogenase pelleted activity remained in the overlay and showed no sign of distribution throughout the gradient as shown in (fig.1.7).

Proteins distribution pattern:-

proteins distributed broadly in the gradient with a peak at 37-38% w/w sucrose in a position between the mitochondrial marker peak and the lysosomal marker (fig.1.8). The amount of protein which appeared in the cushion might represents the intact cells and cells debris which escaped the low speed centrifugation, or more likely due to the interference of the high concentration of sucrose at this part of the gradient (65% w/w), which escaped the wash after the precipitation of protein, because this part of the gradient did not show any enzyme activity.

Cardiac glycoside accumulation and release:-

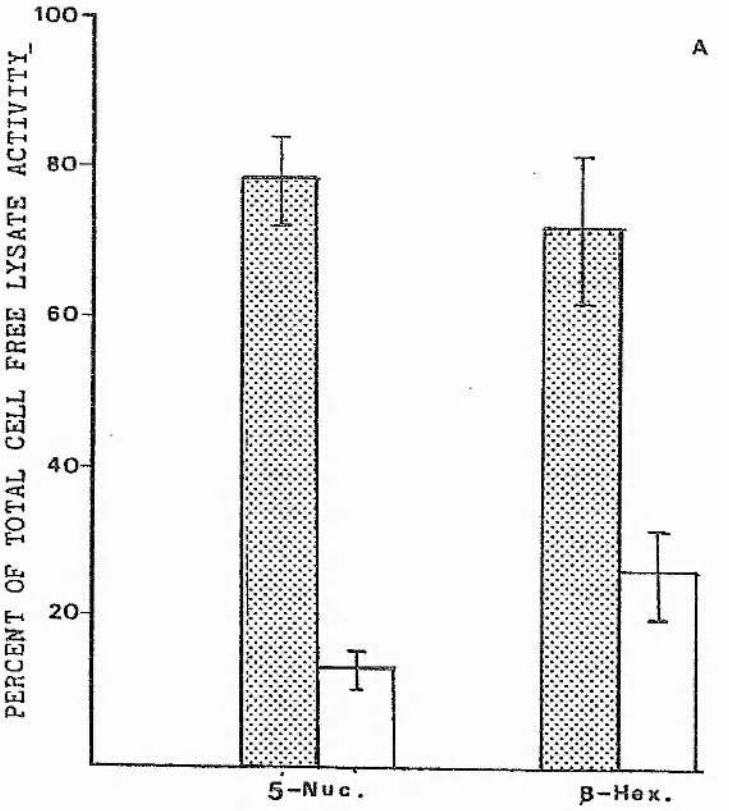
HeLa cells grown for 48 hours in the presence of low concentration, (1X1E-08 M), of cardiac glycoside accumulate more ouabain than it did when digoxin was used,(fig1.9). This result is expected because the excretion rate of ouabain from cells loaded for 20 minutes in K free krebs with 2X1E-07 M [3H]ouabain was much slower than that of digoxin loaded cells (fig.1.10,a and b). The previous results were consistent with those described by Griffiths, Lamb, and Ogden (1983); they also found that the rate of

recovery of sodium pumps blocked with ouabain or digoxin was the same, and almost equal to the excretion rate of digoxin. ouabain on the other hand was found to have a much slower rate of excretion.

Fig.1.4,a and b : The amount of enzyme activity recovered in the particulate fraction.

The activity of the relevant markers was measured in the particulate fraction (dotted column), and in the cytosolic fraction (clear column). The recovered activity in each fraction is plotted as the percent of total cell free lysate activity. Fig.1.4,a shows the result of 5'-nucleotidase (5'-nuc), and B-hexosaminidase (B-hex). Fig.1.4,b shows the activity of lactate dehydrogenase (LDH), and succinate dehydrogenase (SDH). The results are the mean of three experiments ± S.D.

(fig.1.4, a&b)


A
PERCENT OF TOTAL CELL FREE LYSATE ACTIVITY
100
80
60
40
20
5-Nuc.
β-Hex.

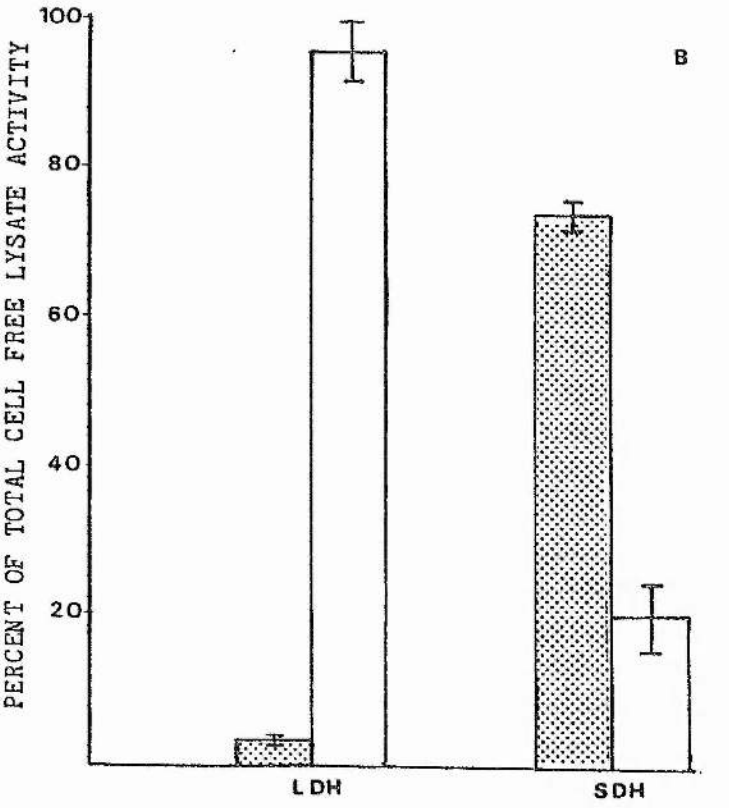

B
PERCENT OF TOTAL CELL FREE LYSATE ACTIVITY
100
80
60
40
20
LDH
SDH

Fig.1.5 : The distribution pattern of 5'-nucleotidase.

The particulate fraction was prepared and then layered on the linear sucrose gradient. The enzyme activity was detected in each fraction and plotted as the percent of the peak fraction which was at 30-31% w/w sucrose. Little activity was found in the overlay. Other relevant markers peak activity fraction are indicated by arrows. (MIT) succinate dehydrogenase, (LYS) B-hexosaminidase, (Y) lactate dehydrogenase. The points are the mean of three gradiens.

(fig.1.5)

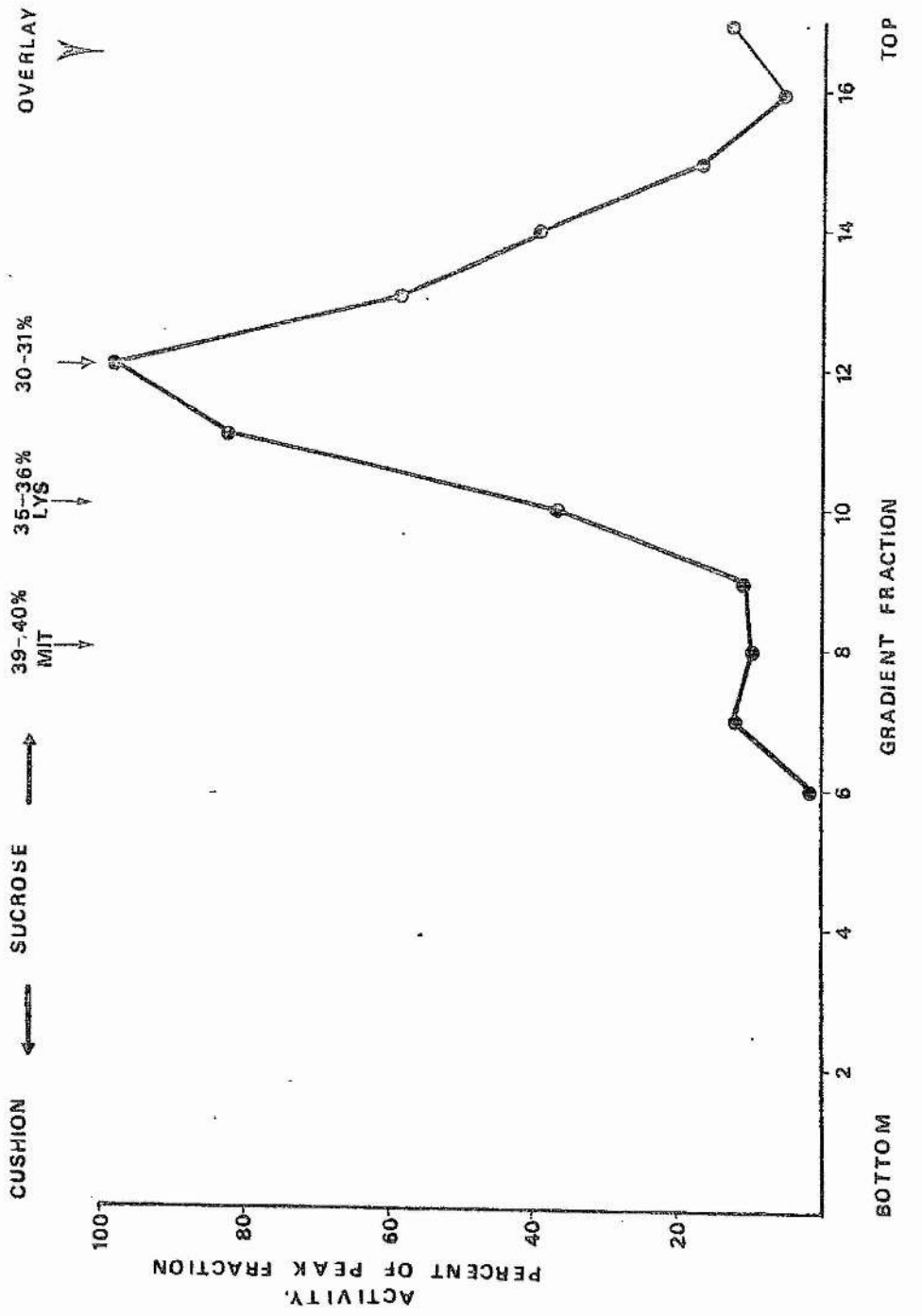

CUSHION
SUCROSE
39-40% MIT
35-36% LYS
30-31%
OVERLAY
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Fig.1.6 : The distribution pattern of B-hexosaminidase.

The enzyme distributed broadly throughout the gradient and peaked at 35- 36% w/w sucrose with a substantial amount of activity remained in the overlay. The conditions and symbols are similar to that of fig.1.5.

(fig.1.6)

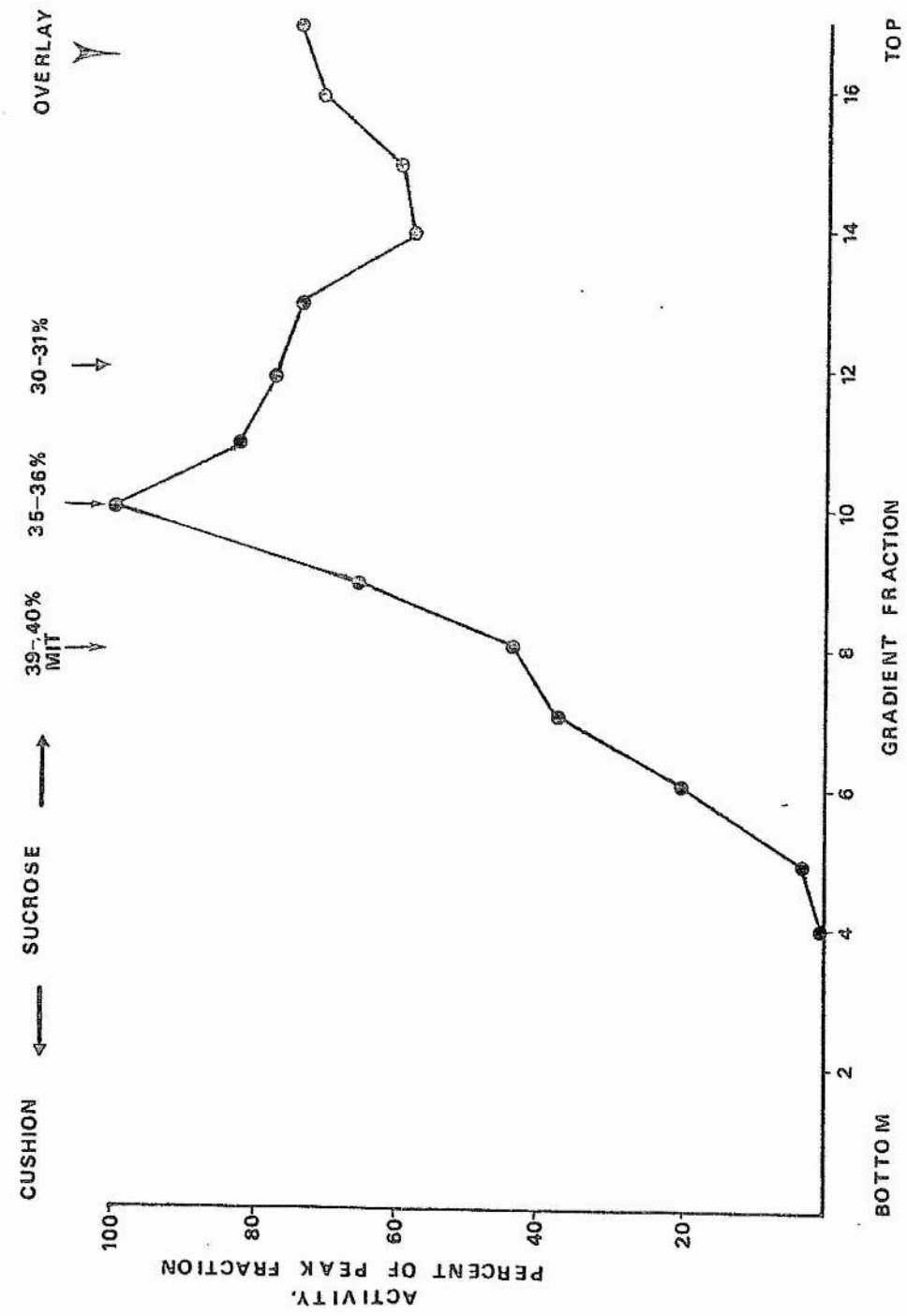

CUSHION
SUCROSE
39-40% MIT
35-36%
30-31%
OVERLAY
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Fig.1.7 : The distribution pattern of succinate dehydrogenase, and lactate dehydrogenase.

All the succinate dehydrogenase pelleted activity entered the gradient and showed a limited distribution pattern with a peak of activity in the dense part of the gradient at 39-40% w/w sucrose. (Y) shows the lactate dehydrogenase activity. All the pelleted activity of this cytosolic marker remained in the top of the gradient. Conditions and symbols are similar to fig.1.5 .

(fig.1.7)

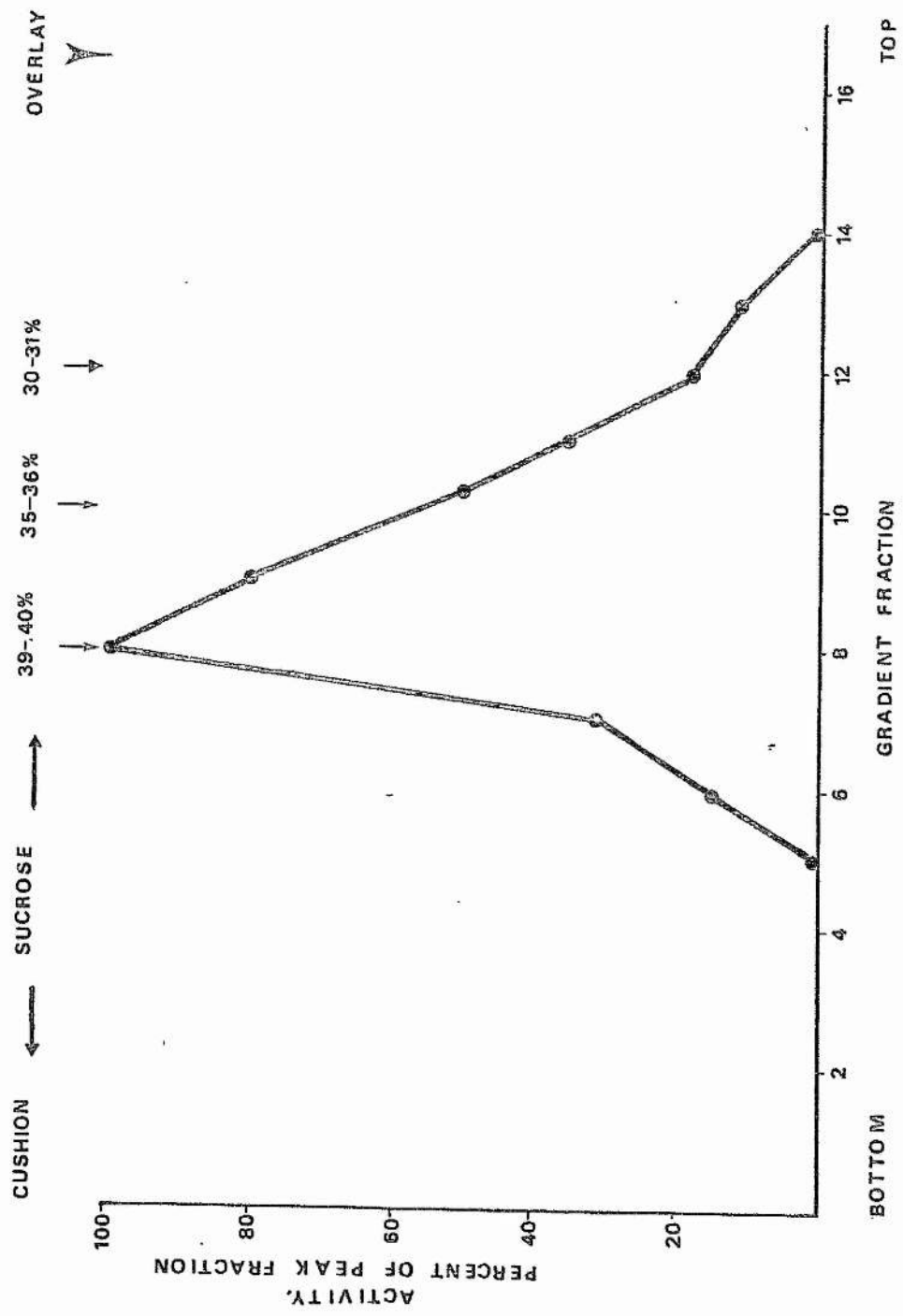

OVERLAY
30-31%
35-36%
39-40%
SUCROSE
CUSHION
ACTIVITY,
PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Fig.1.8 : The distribution pattern of proteins.

The amount of protein in each gradient fraction was determined and was found to have a broad distribution throughout the gradient, and peaked in the 9th fraction with some activity at the bottom of the gradient (cushion). The conditions and symbols are similar to that of fig.1.5.

(fig.1.8)

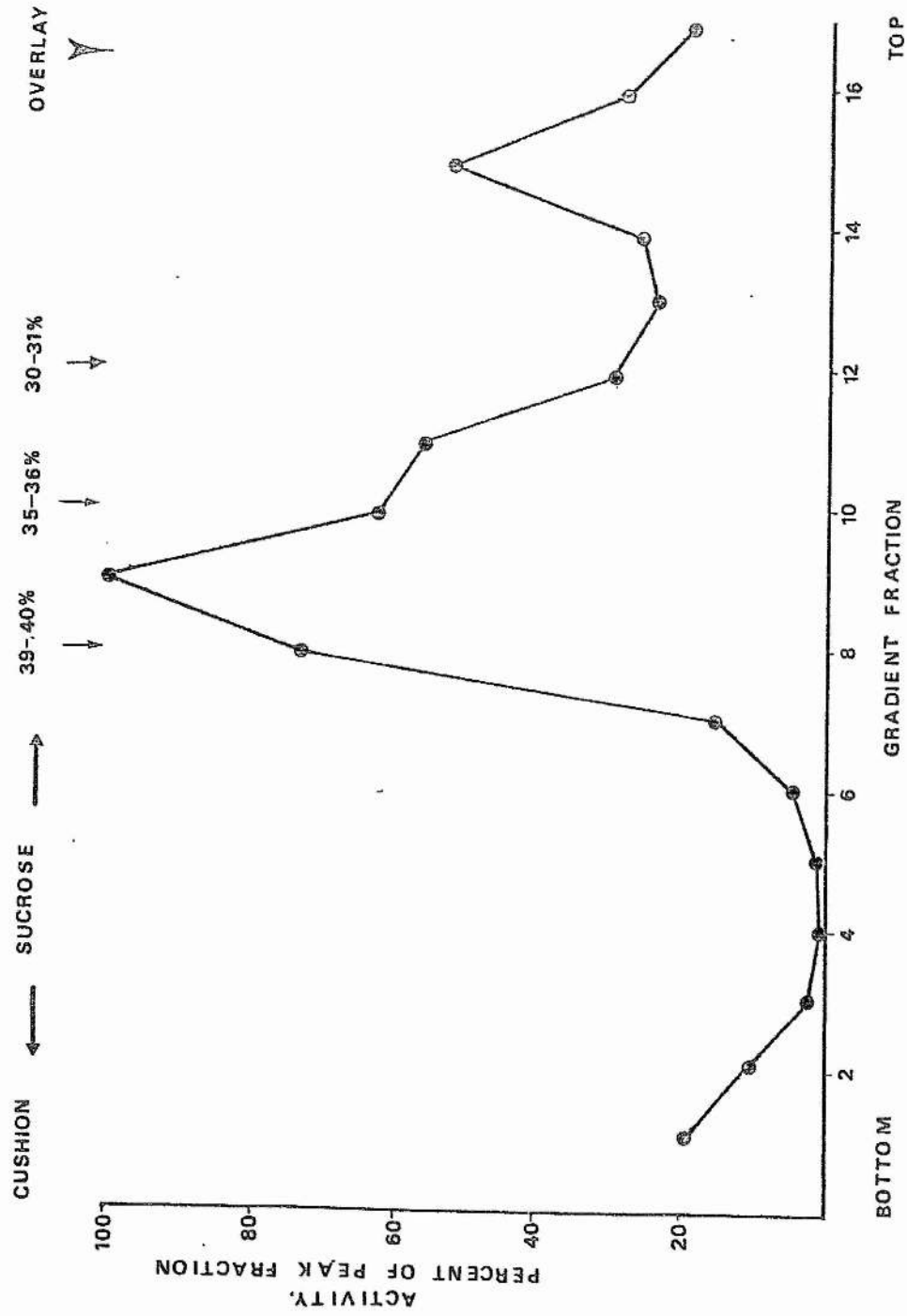

CUSHION
SUCROSE
39-40%
35-36%
30-31%
OVERLAY
ACTIVITY,
PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Fig.1.9 : The accumulation of ouabain and digoxin in HeLa cells.

Cells were grown for 48 hours in the presence of 1X1E-08 M [3H] ouabain or digoxin. It was found that cells accumulate more ouabain (clear column), than digoxin (dotted column). The results are the mean of three experiments ± S.D. calculated as n moles/mg protein.

(fig.1.9)

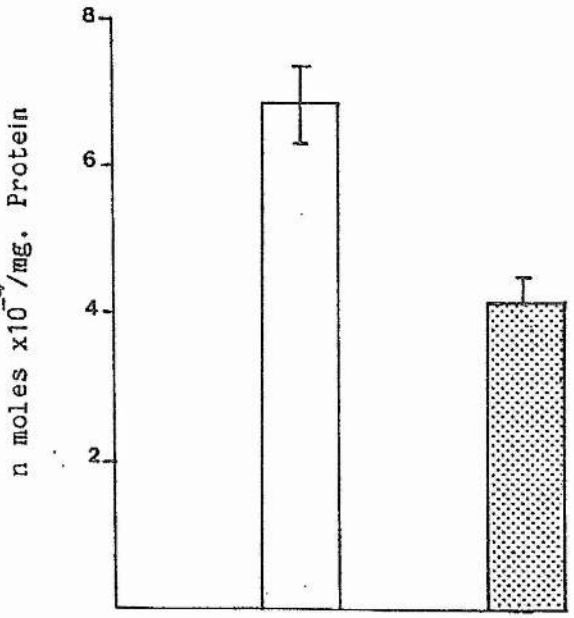
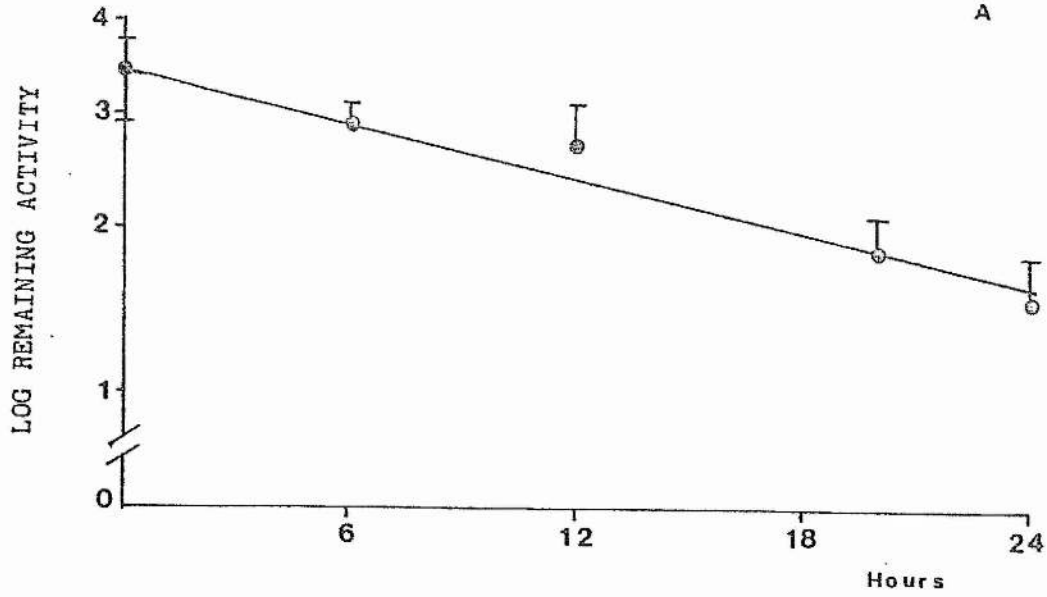
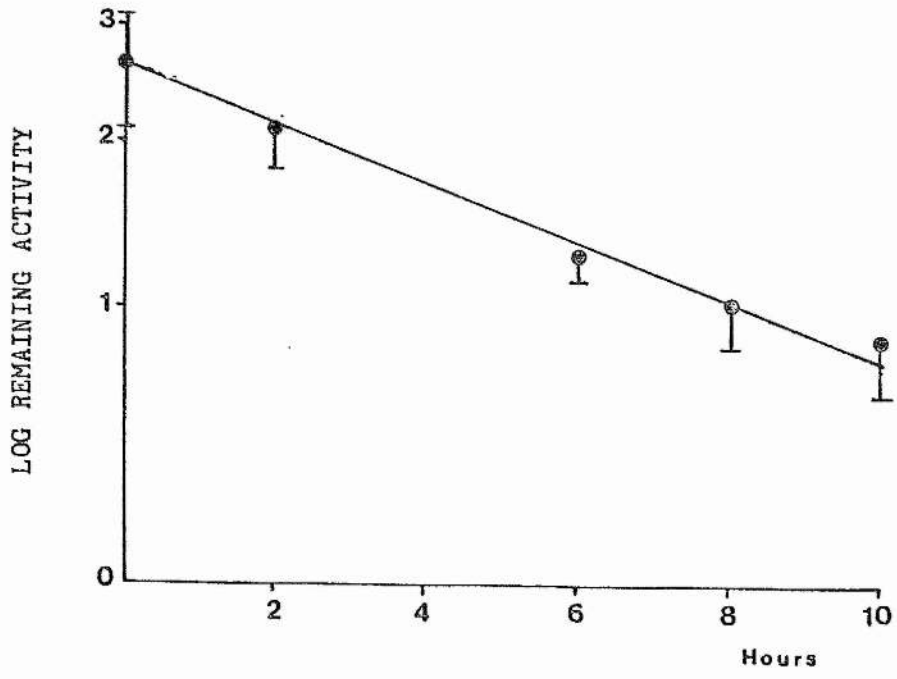


Fig.1.10,a&b : The excretion rate of ouabain and digoxin.

cells were loaded with 2X1E-07 M [3H] ouabain or digoxin, washed and returned to growth medium. At times shown, the remaining activity per mg protein was estimated and plotted against time on a semilograthmic scale. The best fitting line (by eye), shows that ouabain has a slow excretion rate (t1/2= 22 hours). While digoxin was excreted in a much faster rate (t1/2= 6.5 hours). The results are the mean of 3 - 5 experiments ± S.D.

(fig.1.10,a&b)


A
LOG REMAINING ACTIVITY
4
3
2
1
0
6
12
18
24
Hours


B
LOG REMAINING ACTIVITY
3
2
1
0
2
4
6
8
10
Hours

The localization of surface bound and retained glycosides:-

Previous results (Cook, et al 1978), and (Lamb, Ogden 1982) suggested the involvement of ouabain internalization after binding to the HeLa cell surface. Cells were loaded with [3H]-ouabain and either fractioned immediately or returned to normal growth medium for 24hours and then fractioned. Immediately after binding, 85% of the total ouabain activity was recovered in the particulate fraction and 17% remained in the cytosolic fraction . 24 hours after binding, less activity was recovered in the particulate fraction (70%), and more in the cytosolic fraction (23%), (fig.1.11,a). At 0 time ouabain showed a distribution pattern throughout the linear sucrose gradient similar to that of the plasama membrane marker, 5'-nucleotidase (fig.1.12); but 24 hours later ouabain co-distributed with the lysosomal marker, B-hexosaminidase showing similar peak, distribution, and a substantial amount of activity in the overlay (fig.1.13).

Different results were obtained when digoxin was used. The percentage of total cell free lysate activity recovered in the particulate fraction and that remained in the cytosolic fraction were unchanged in samples analysed immediately after digoxin binding or 9 hours later (fig.1.11,b).

Fig.1.11,a&b : The amount of ouabain or digoxin recovered in the particulate fraction.

The amount of activity recovered in the particulate fraction (dotted column), and that remained in the cytosolic fraction (clear column) of ouabain (fig.1.11,a), or digoxin (fig.1.11,b), was measured immediately after binding or 24 hours later (ouabain) or 9 hours later (digoxin). Ouabain activity recovered in the particulate fraction was decreased after internalisation and that remained in the cytosolic fraction was increased. While digoxin activity did not change in both cases. The results are the mean of three experiments plotted as the percent activity recovered of the total cell free lysate ± S.D.

(fig.1.11,a&b)

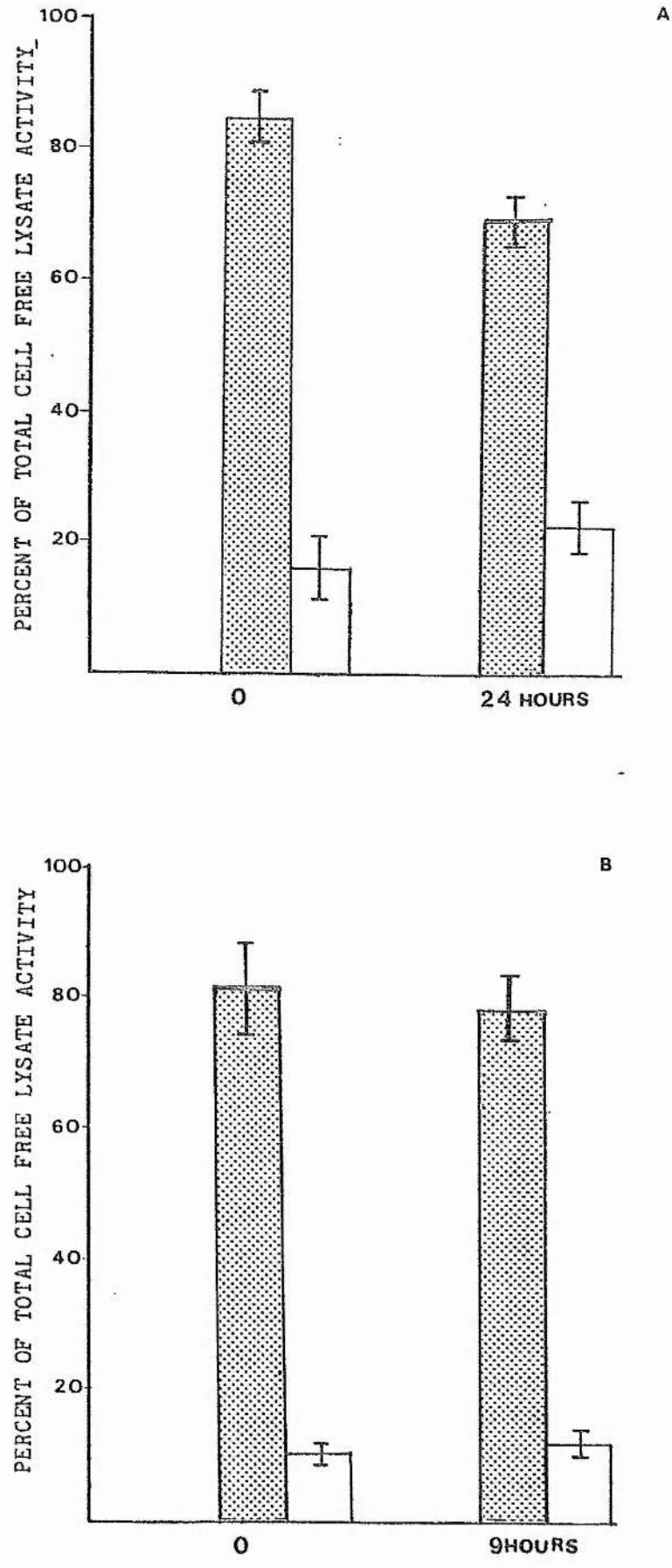

A
PERCENT OF TOTAL CELL FREE LYSATE ACTIVITY
100
80
60
40
20
0
24 HOURS
B
PERCENT OF TOTAL CELL FREE LYSATE ACTIVITY
100
80
60
40
20
0
9HOURS

Fig.1.12 : The distribution pattern of ouabain activity immediately after binding.

Cells were loaded with ouabain for 20 min, washed for 15 min, and the particulate fraction was prepared and then layered on the linear sucrose gradient. Ouabain (open circles) has distributed in a pattern similar to that of 5'-nucleotidase (- - -), but different from that of B-hexosaminidase (closed circles). The arrows shows the peak activity fraction of other relevant markeres. (MIT) succinate dehydrogenase, (Y) lactate dehydrogenase. Points are the mean of three gradients plotted as the percent of peak activity fraction.

(fig.1.12)

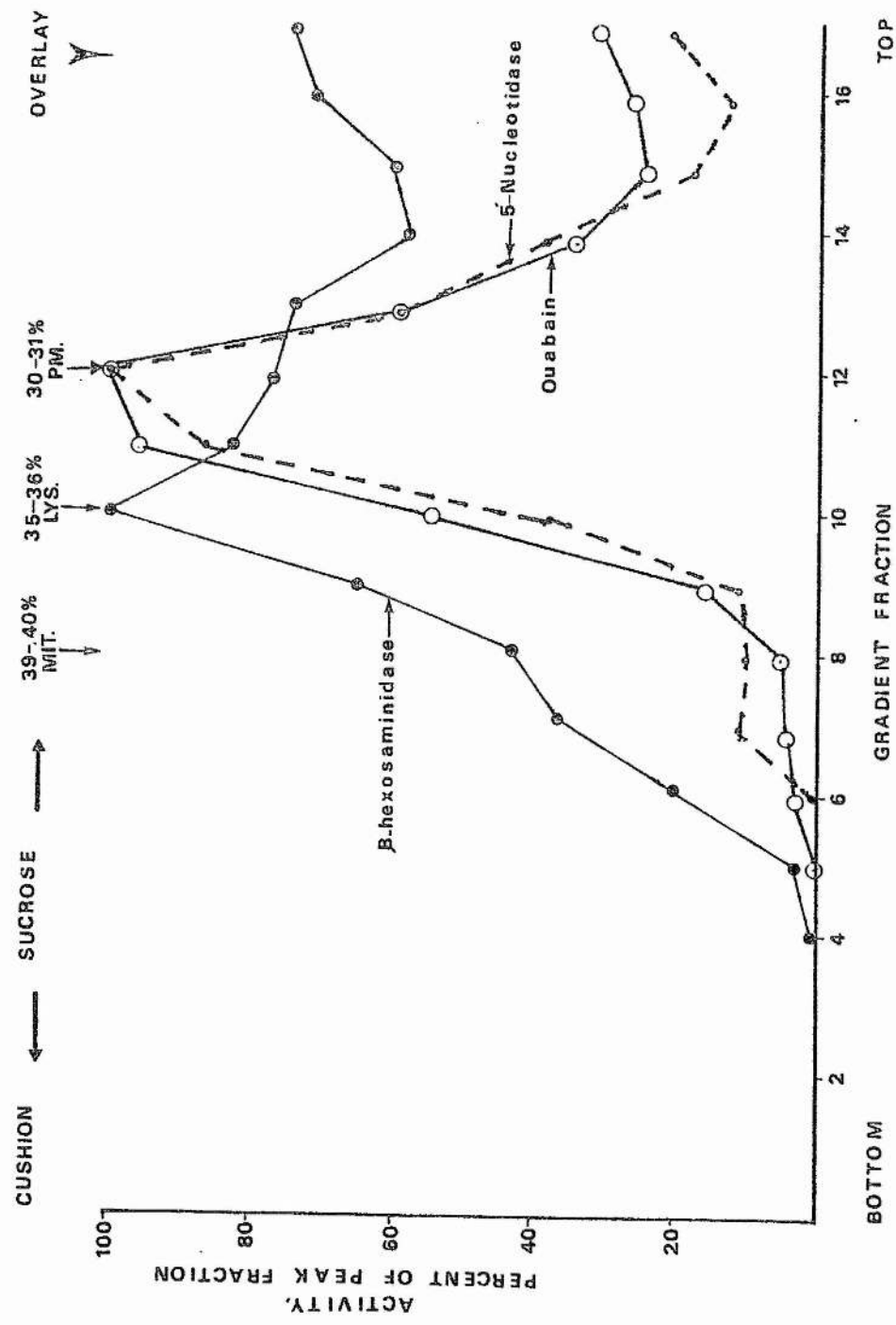
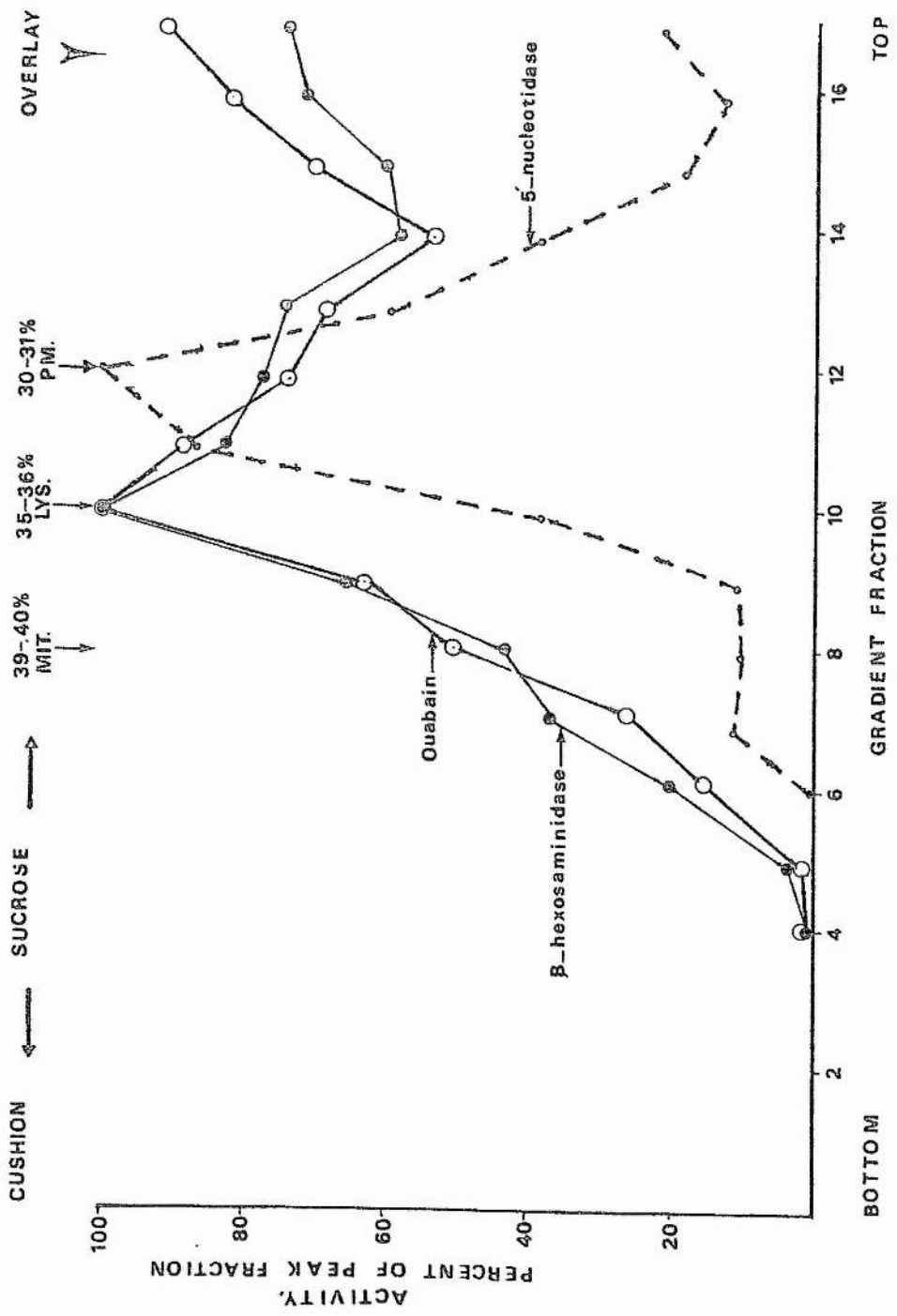

OVERLAY
CUSHION
SUCROSE
30-31% PM.
35-36% LYS.
39-40% MIT.
5-Nucleotidase
Ouabain
β-hexosaminidase
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Fig.1.13 : The distribution pattern of internalised ouabain.

Cells were loaded with ouabain, washed, and returned to normal growth medium for 24 hours. The particulate fraction was prepared and then layered on the sucrose gradient. Ouabain (open circles) showed a distribution pattern different from that obtained immediately after binding, but similar to that of B-hexosaminidase (closed circles), with a substantial amount of activity in the overlay. This distribution was also different from that of the 5'-nucleotidase (- - -). Symbols and conditions are similar to that of fig.1.12.

(fig.1.13)


OVERLAY
CUSHION
SUCROSE
30-31% PM.
35-36% LYS.
39-40% MIT.
Ouabain
β_hexosaminidase
5'_nucleotidase
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

Digoxin immediately after binding to HeLa cells was found on the cell surface (fig.1.14), but 9 hours later it was no longer co-distributing with 5'-nucleotidase. The distribution pattern showed the peak activity at 35-36% w/w sucrose, the lysosomal marker peak, but the distribution pattern throughout the sucrose gradient did not mimic the whole distribution of B-hexosaminidase (fig.1.15), specially the lack of activity on the overlay. It also did not mimic the distribution of any other relevant marker. When digitoxin was used the result was similar to that of digoxin (fig.1.16).

The cardiac glycosides activity which enters the gradient resembles a specifically bound or internalized activity. This was shown by adding an amount of ouabain or digoxin activity equal of that usually found in the particulate fraction immediately after binding, to a control particulate fraction, and then layered on the gradient. Both ouabain and digoxin activities remained in the overlay (fig.1.17,a&b). This eliminate the possibility of digoxin or ouabain being diffused to the particulate fraction. This is important for digoxin is known to have a lipophilic property. Moreover when a control particulate fraction was incubated at 37°c for 20 minutes in the presence of 2X1E-07 M ouabain or digoxin, repelleted, and then layered on the gradient, no sign of binding was detected in this sample.

Fig.1.14 : The distribution pattern of digoxin immediately after binding.

Cells were loaded with digoxin for 20 min, washed for 15 minutes, and the particulate fraction was prepared then layered on the sucrose gradient. Digoxin (open circles) showed a distribution pattern similar to that of 5'-nucleotidase (- - -). B-hexosaminidase distribution pattern (closed circles), and other markers peak activity are represented by arrows. (MIT) succinate dehydrogenase, (Y) lactate dehydrogenase. The results are the mean of three gradients plotted as the percent of peak activity.

(fig.1.14)

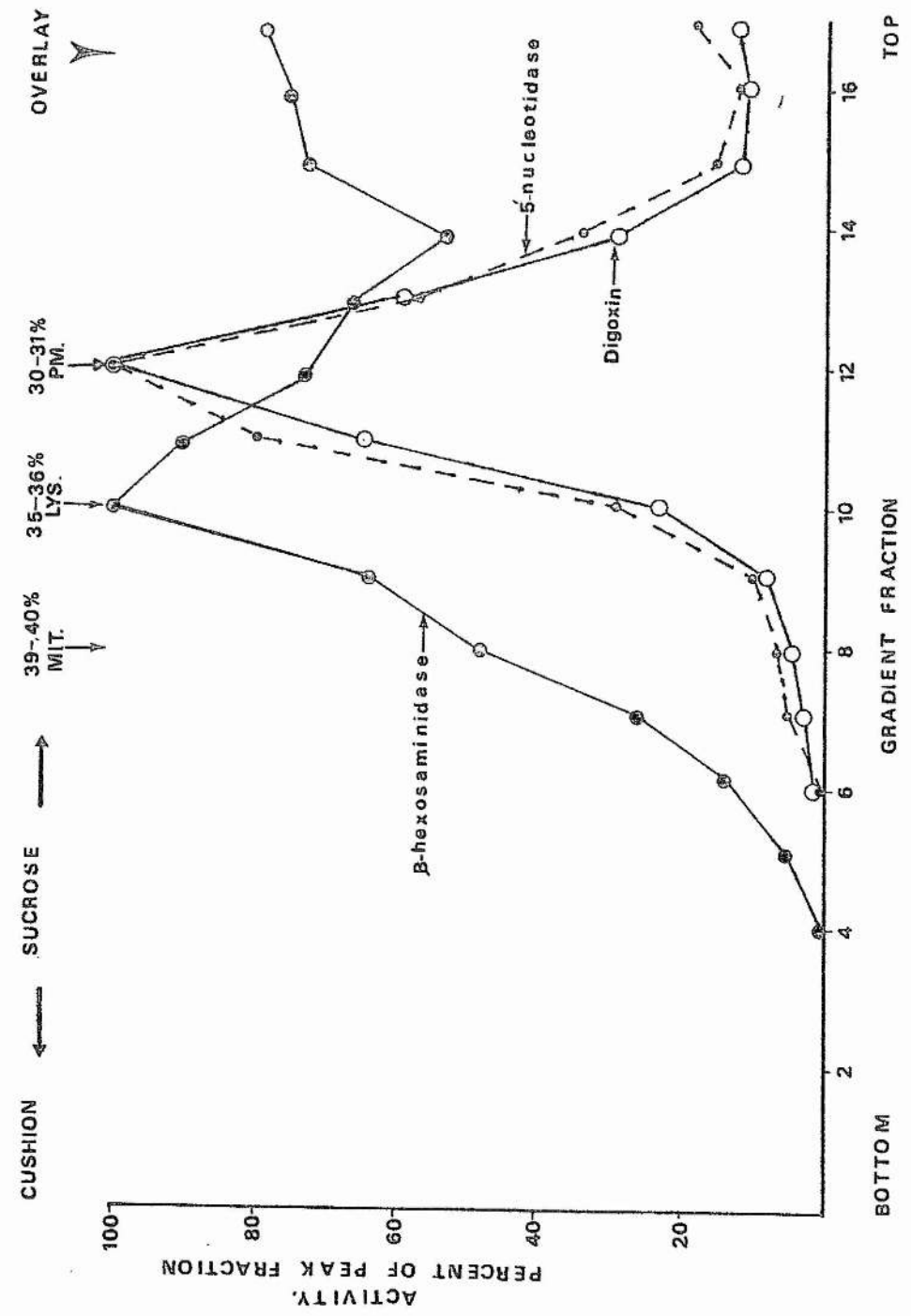

OVERLAY
CUSHION
SUCROSE
30-31% PM.
35-36% LYS.
39-40% MIT.
5-nucleotidase
Digoxin
β-hexosaminidase
ACTIVITY
PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.1.15 : The distribution pattern of internalised digoxin.

Cells were loaded with digoxin for 20 min, washed and returned to normal growth medium for 9 hours. On the gradient digoxin (open circles) showed a peak of activity in a position similar to that of B-hexosaminidase (closed circles), but did not mimic the whole distribution. Also the overlay was clear from substantial activity. But this distribution was significantly different from that of the 5'-nucleotidase (- - -). Symbols and conditions are similar to that of fig.1.14.

(fig.1.15)

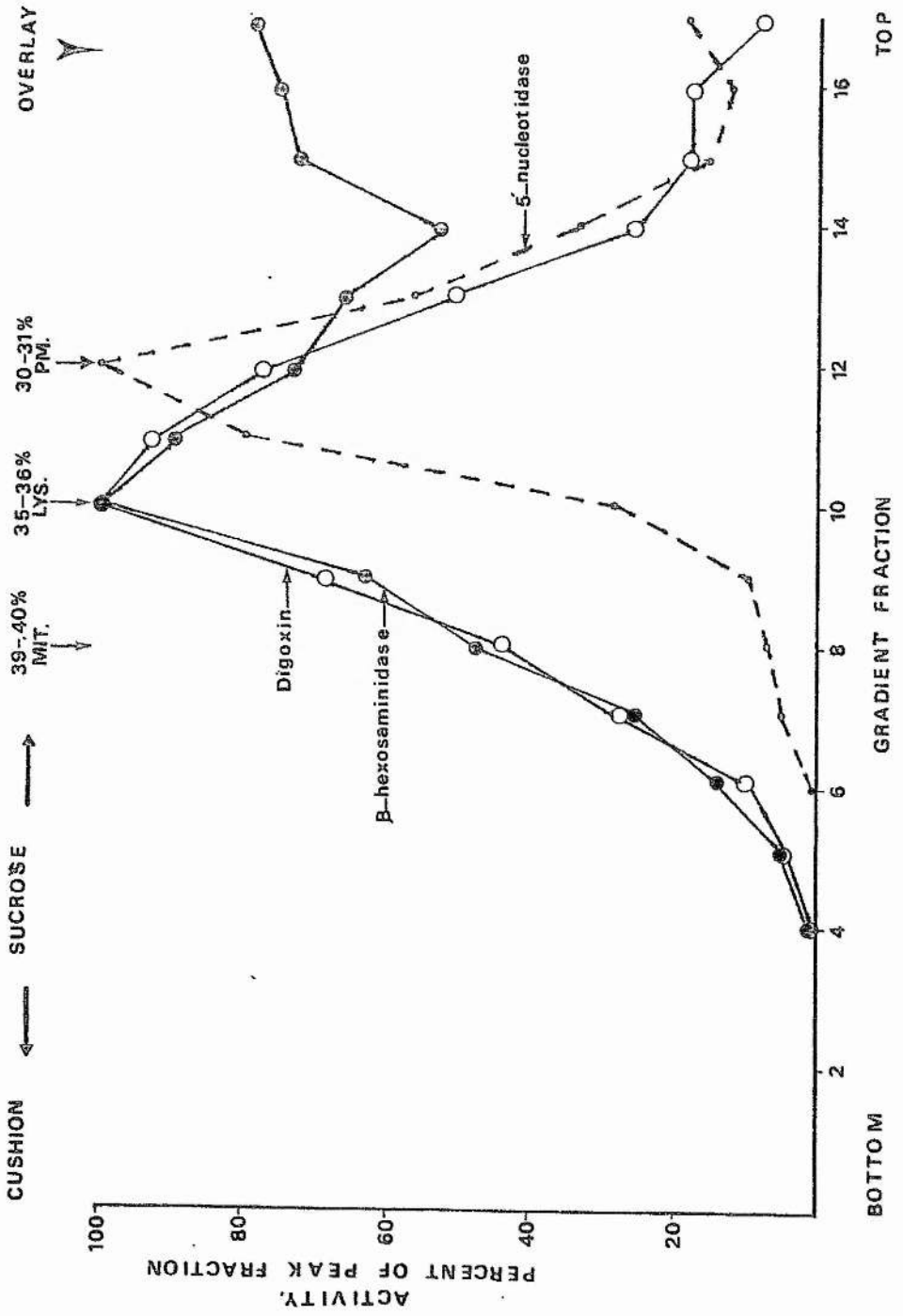

OVERLAY
30-31% PM.
35-36% LYS.
39-40% MIT.
CUSHION
SUCROSE
Digoxin
β-hexosaminidase
5'-nucleotidase
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.1.16 : Digitoxin distribution patterns.

The distribution pattern of digitoxin in the linear sucrose gradient immediately after binding (open circles), and 9 hours after binding (triangles), compared to the distribution of 5'-nucleotidase (- - -), and B-hexosaminidase (closed circles). Other relevant markers peak activity are indicated by arrows. (MIT) succinate dehydrogenase, (Y) lactate dehydrogenase. The results are the mean of two gradients.

(fig.1.16)

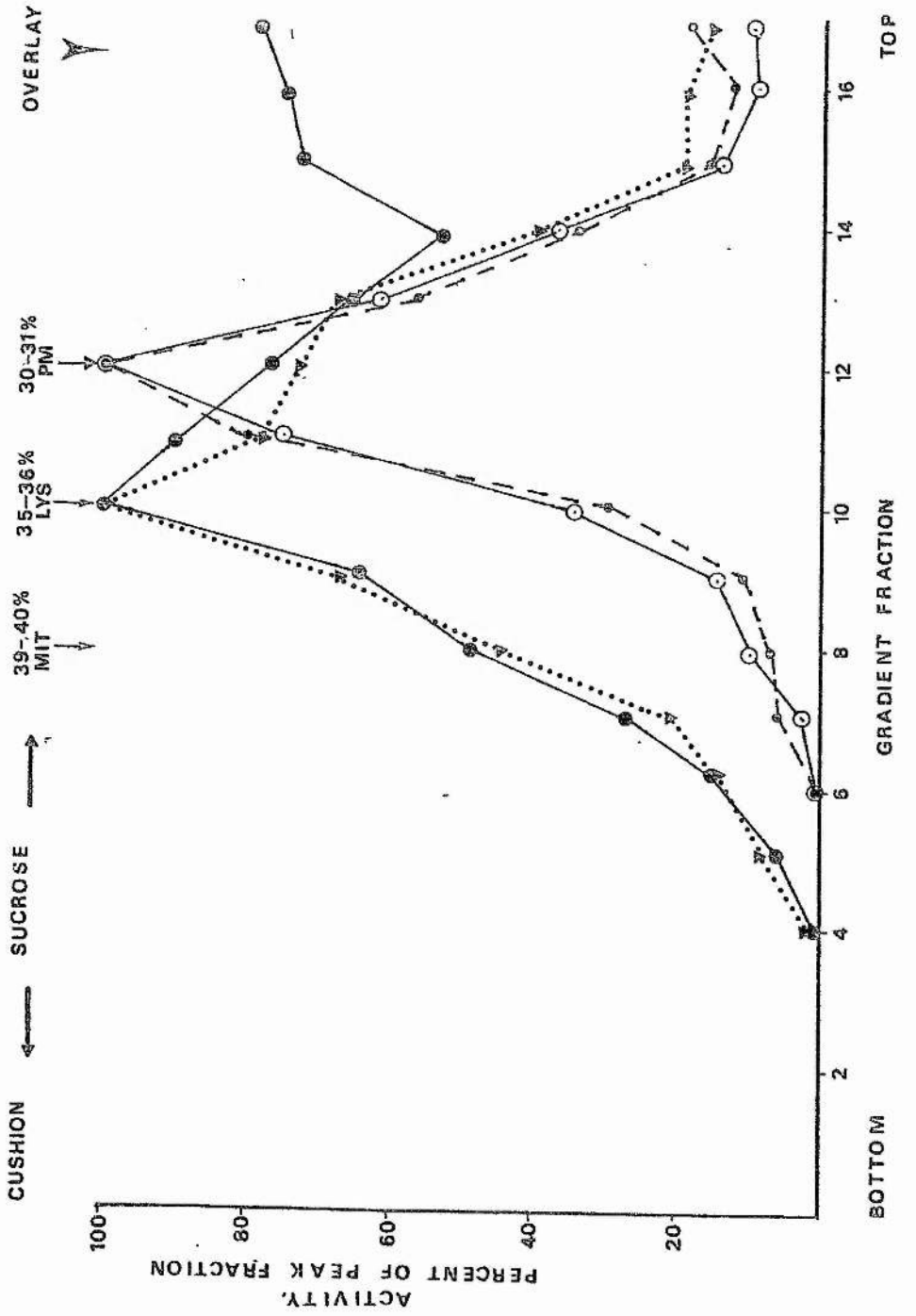
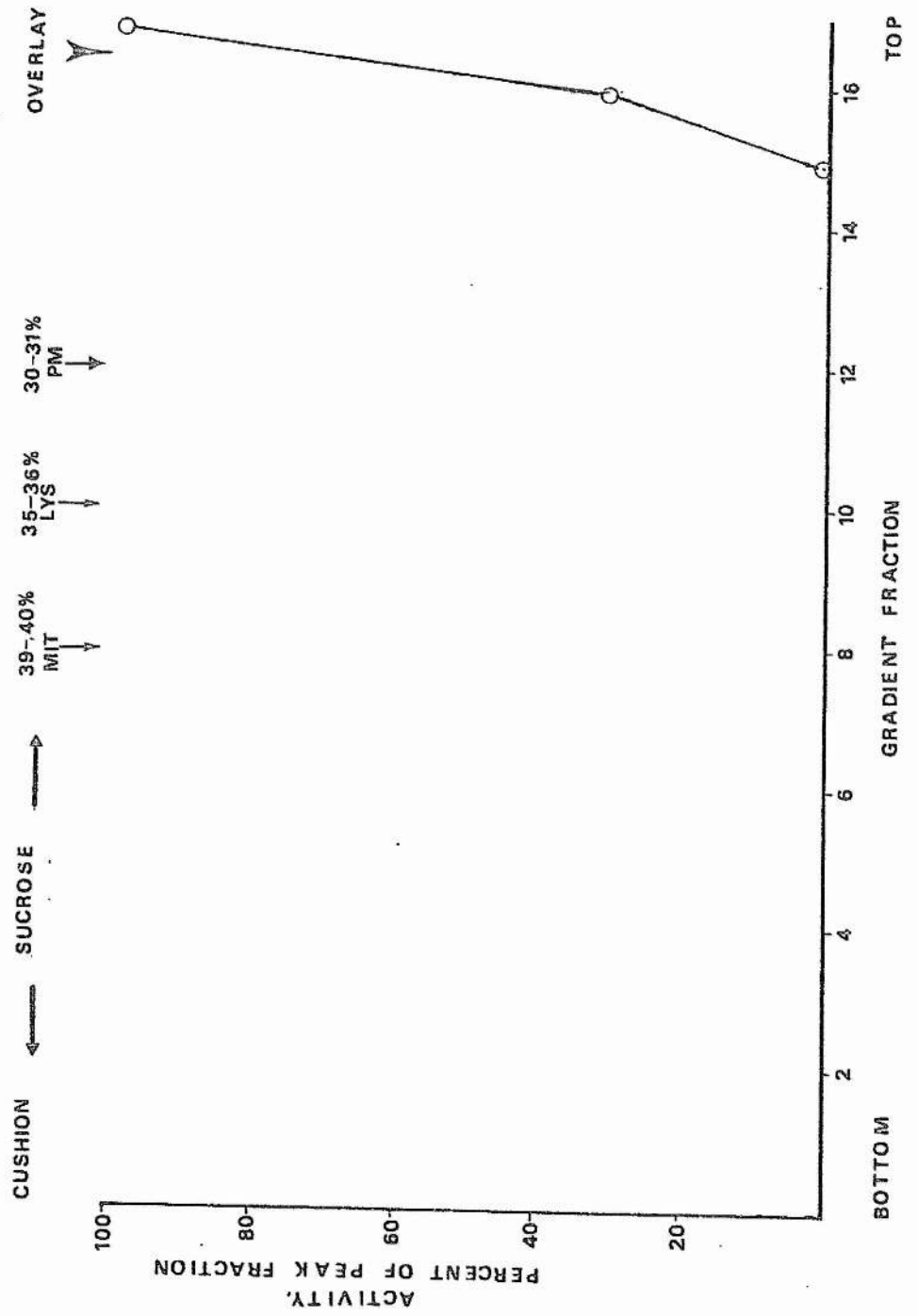

OVERLAY
30-31% PM
35-36% LYS
39-40% MIT
CUSHION
SUCROSE
BOTTOM
TOP
GRADIENT FRACTION
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16

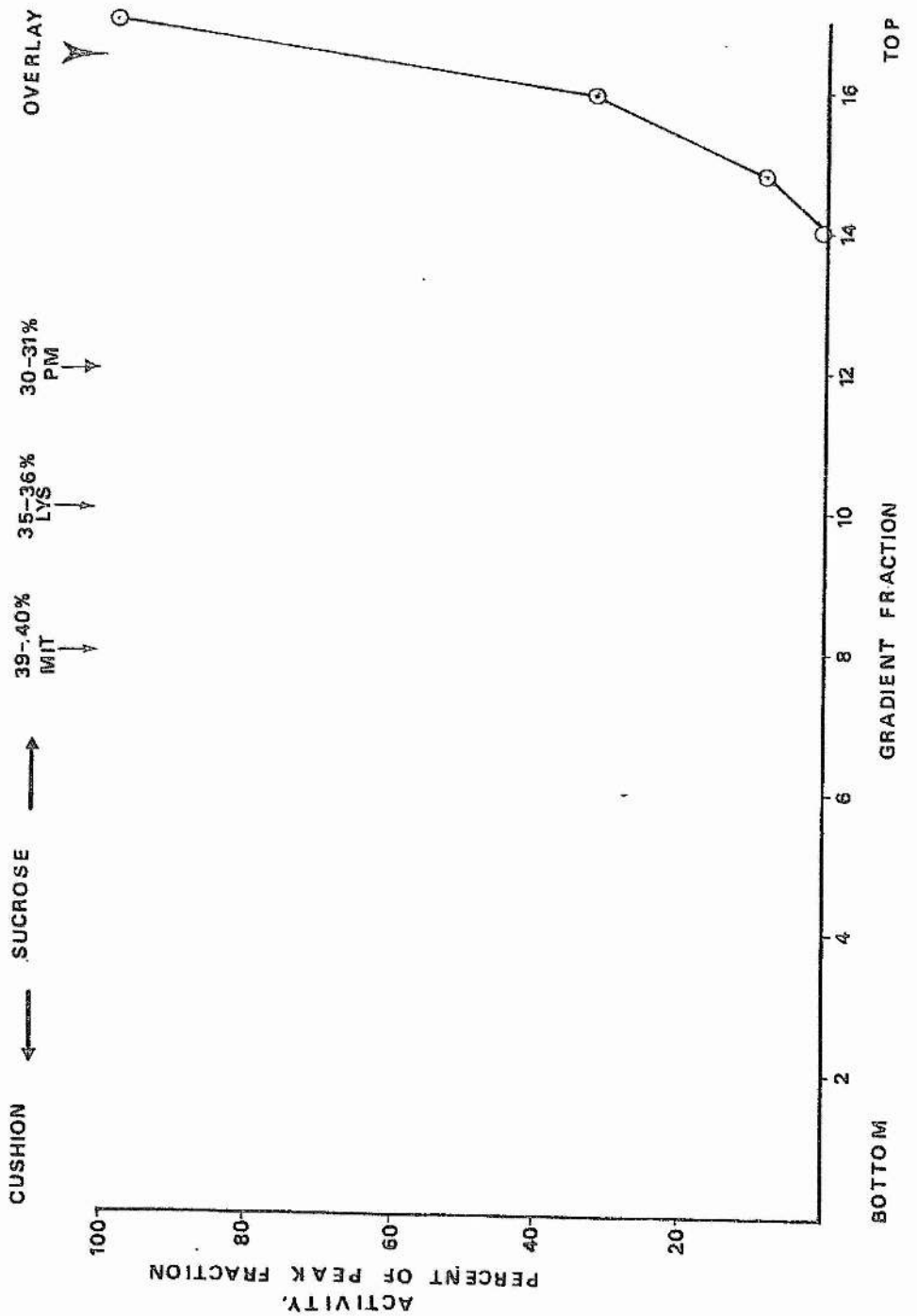
Fig.1.17,a&b : The fate of unbound ouabain or digoxin activity.

An equal amount of either ouabain or digoxin activity usually found in the particulate fraction of loaded cells, was added to a control particulate fraction, and then layered on the gradient. All the activity of both ouabain (fig.a), and digoxin (fig.b), remained on the overlay and did not enter the gradient. The results are the mean of three gradients for each experiment. Arrows represent the peak activity fraction of the four markeres. (MIT) succinate dehydrogenase, (LYS) B-hexosaminidase, (PM) 5'-nucleotidase, and (Y) lactate dehydrogenase.

(fig.1.17,a)


OVERLAY
30-31% PM
35-36% LYS
39-40% MIT
CUSHION
SUCROSE
BOTTOM
TOP
GRADIENT FRACTION
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16

(fig.1.17,b)


OVERLAY
30-31% PM
35-36% LYS
39-40% MIT
CUSHION
SUCROSE
TOP
BOTTOM
GRADIENT FRACTION
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16

The previous results demonstrate that non specific or indeed specific binding does not occur in this preparation perhaps partly due to the lack of ATP. In other experiment when ouabain was bound to the cells in 15 mM K Krebs (non specific binding), only 7% of the total binding occur, when the radioactivity was measured in the sample, but the distribution pattern of the particulate fraction was undetectable.

The effect of severe homogenization on the release and recovery of internalized ouabain and digoxin:-

In order to release the content of the lysosomes, one of two methods can be used. Either by continuous freezing and thawing of the lysosomal preparation, or by using severe homogenization. If severe homogenization (100 Dounce homogenizer strokes), was applied to the cell suspension in order to disrupt most of the lysosomes, and then the cell free lysate (F-I) was layered on the gradient. B-hexosaminidase activity distributes throughout the gradient in a way different from that obtained when the particulate fraction (P-II) was prepared using normal homogenization (40 strokes). Most of the released activity remained in the overlay (fig.1.19), and very little activity enters the gradient. This is expected because the application of severe homogenization release most of the B-hexosaminidase activity as a result of ruptured lysosomes.

Similar treatment for cells loaded with ouabain and returned to normal growth medium for 24 hours gave a matching distribution pattern (fig.1.18). On the other hand digoxin retained in the cells 10 hours after binding showed a different distribution pattern (fig.1.19), but it was similar to that obtained from cells homogenized with 40 strokes showing a peak of activity at 35 - 36% w/w sucrose.

Cook and Brake (1978), described a homogenizing technique (shear sensitivity analysis), which was used to illustrate that internalized ouabain was released by homogenization from the particulate fraction with same kinetics as B-hexosaminidase. The same technique was applied to cells 10 hours after loading with digoxin. As homogenization increases the quantity of both B-hexosaminidase and digoxin activities that can be released into the cell free lysate (F-I) increases too (fig.1.20,a). The amount of B-hexosaminidase activity that can be sediment in the particulate fraction also increases as homogenization increases up to 40 strokes reaching a maximum of 62% of the total cell homogenate activity (fig.1.20,b). But further homogenization leads to decline in the amount of activity that can be sediment in the particulate fraction (fig.1.20,b). On the other hand the quantity of digoxin activity reached a maximum of 56% with 40 strokes and remained steady with further homogenization (fig.1.20,b).

Fig.1.18 : The effect of severe homogenization on the release of both B-hexosaminidase and ouabain activities and their distribution patterns.

Cells were loaded with ouabain, washed and returned to normal growth medium for 24 hours. The cell free lysate (F-I) was prepared after applying severe homogenization (100 strokes), to the cell suspension. Then it was layered on the linear sucrose gradient, and both B-hexosaminidase and ouabain activities in each gradient fraction were detected. The distribution patterns show that both activities were solubilized by severe homogenization and most of B-hexosaminidase (closed circles), and ouabain (open circles) activities remained in the top of the gradient giving a different distribution patterns from those obtained with normal homogenization. The results are the mean of three gradients plotted as the percent of peak activity.

(fig.1.18)

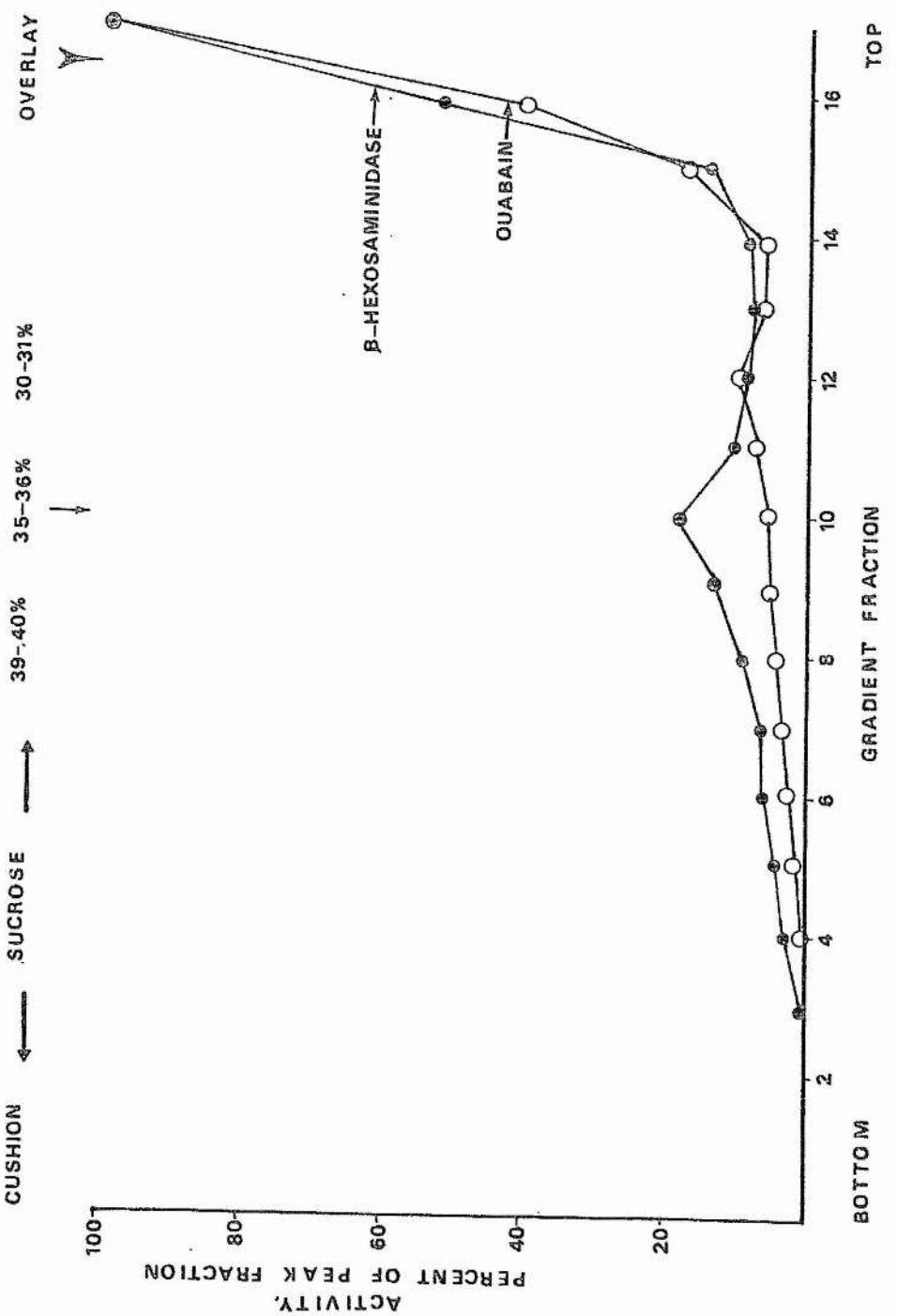
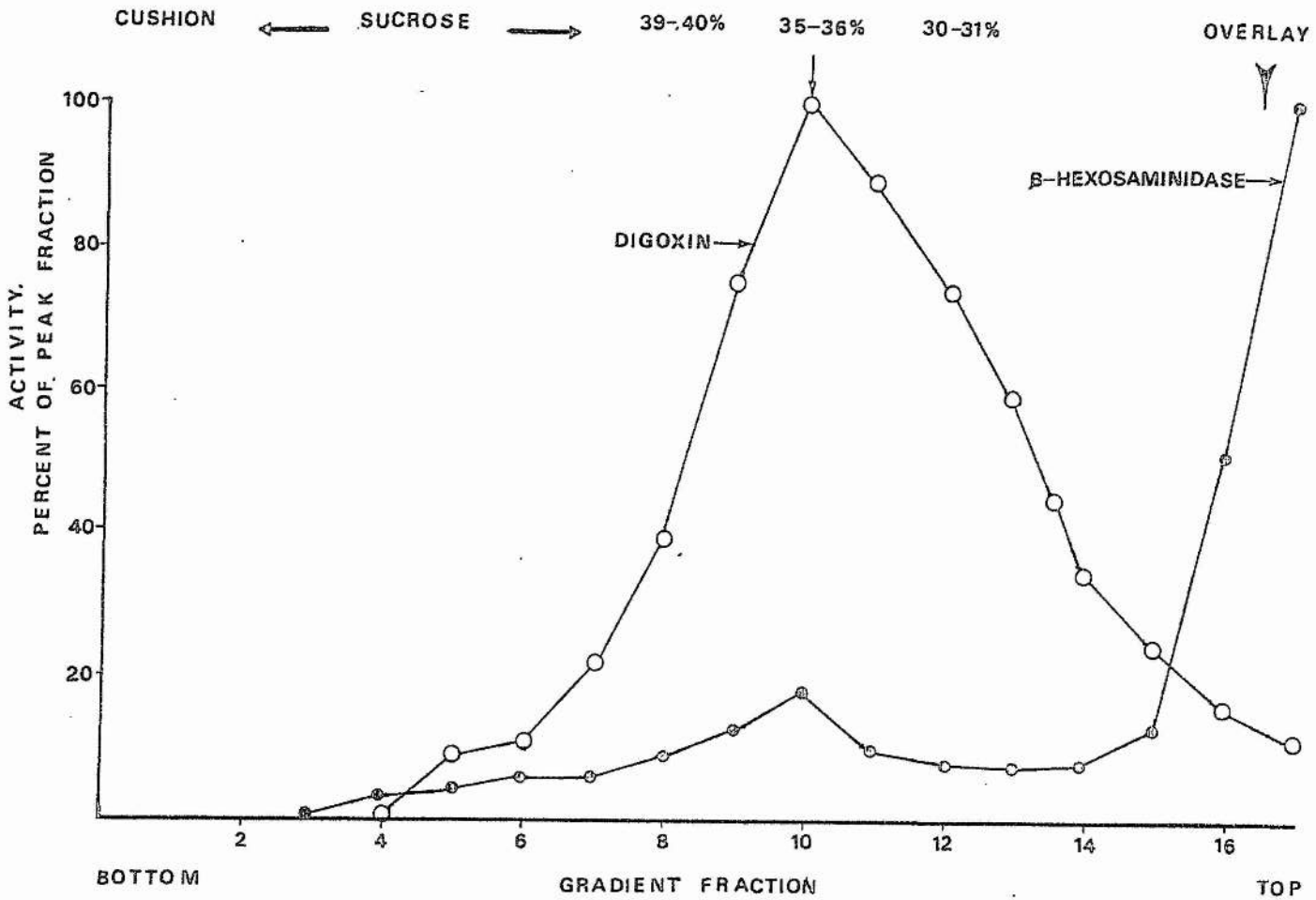

OVERLAY
30-31%
35-36%
39-40%
SUCROSE
CUSHION
β-HEXOSAMINIDASE
OUABAIN
ACTIVITY,
PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.1.19 : The effect of severe homogenization on internalized digoxin and its distribution pattern.

Cells were loaded with digoxin, washed and returned to normal growth medium for 10 hours. The cell suspension was severely homogenized and the cell free lysate was then layered on the sucrose gradient. The distribution pattern of digoxin (open circles) was completely different from that of B-hexosaminidase (closed circles), but it was similar to that obtained from normally homogenized cells when the particulate fraction was layered. Symbols and conditions are similar to that of fig.1.18.

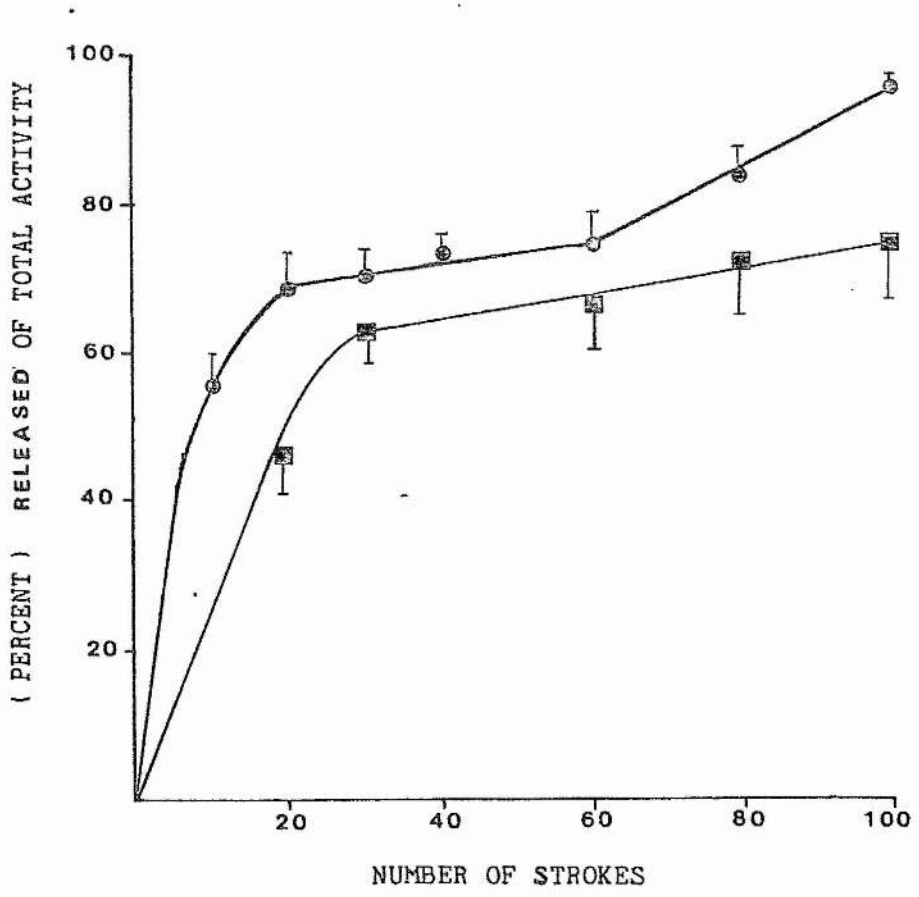

CUSHION
SUCROSE
39-40%
35-36%
30-31%
OVERLAY
β-HEXOSAMINIDASE
DIGOXIN
ACTIVITY.
PERCENT OF PEAK FRACTION
100
80
60
40
20
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

(fig.1.19)

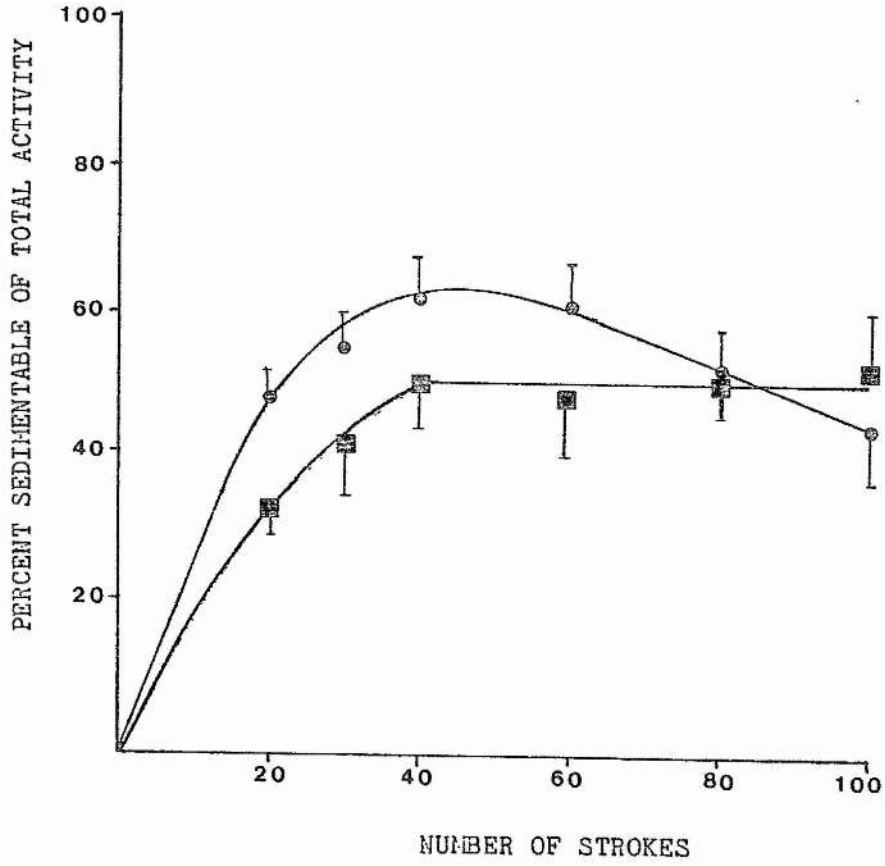
Fig.1.20,a&b : Shear sensitivity analysis of digoxin loaded cells.

Cells were loaded with digoxin, washed and returned to normal growth medium for 10 hours. Cells were then homogenized with an increasing number of strokes. Fig.1.20,a shows the activity of both B-hexosaminidase (closed circles) and digoxin (squares) that has been released into the cell free lysate (F-I) plotted as the percentage of total activity in the original cell homogenate as a function of the number of homogenizing strokes applied to the cell suspension. When the strokes were increased both activities were increased. Fig.1,20,b shows that the activity of B-hexosaminidase which was sedimentable after the centrifugation of the cell free lysate at 22,000 g - 20 min increased reaching a maximum at 40 strokes, further homogenization lead to decline in the activity recovered. Digoxin activity reached the maximum at 40 strokes but further homogenization did not affect the recovered activity. Results are the mean of three experiments ±S.D.

(fig.1.20,a)


(PERCENT) RELEASED OF TOTAL ACTIVITY
100
80
60
40
20
20
40
60
80
100
NUMBER OF STROKES

(fig.1.20,b)


PERCENT SEDIMENTABLE OF TOTAL ACTIVITY
100
80
60
40
20
20
40
60
80
100
NUMBER OF STROKES

The previous results suggests that internalized digoxin is found in a shear insensitive compartment probably the lysosomal membrane while ouabain is retained in the lysosomal interior.

Dextran filled lysosomes:-

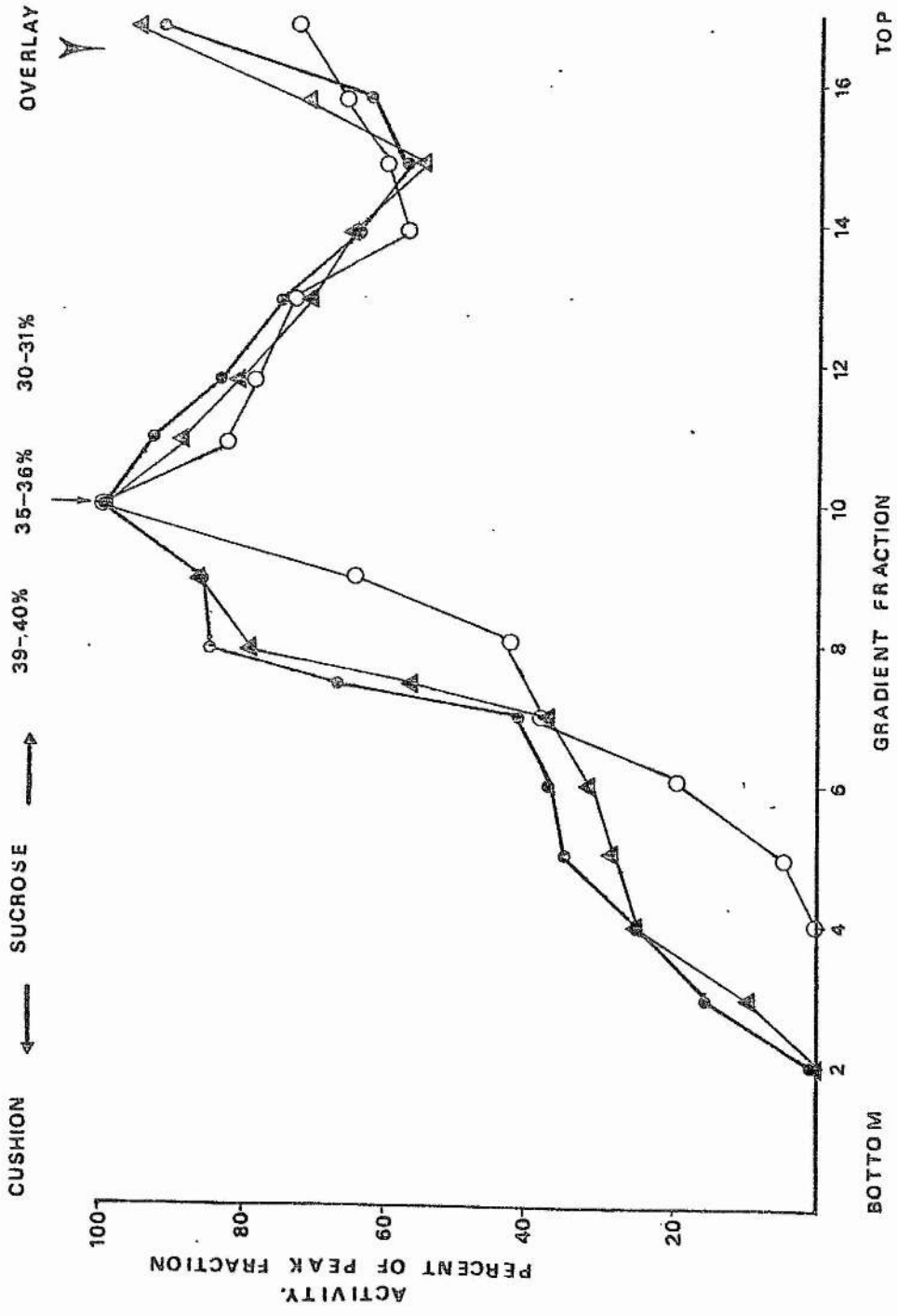
Beaufay, (1972) has described a fractionation method yielding Dextran-500 loaded lysosomes, which takes advantage of an increase of the lysosomal density. On the same bases HeLa cells were grown in the presence of 2% Dextran-500, to obtain a shift in the lysosomal buoyant density towards a heavier density level in the linear sucrose gradient. The aim was to detect whether internalized ouabain or digoxin in dextran treated cells follow the same shift. On the gradient the results were unsatisfactory, as dextran filled lysosomes distributed broadly throughout the gradient if compared with the distribution pattern of control lysosomes. But both distributions showed a peak of activity at 35-36% w/w sucrose (fig.1.21). Ouabain in particulate fraction prepared from dextran treated cells 24 hours after binding was found to have similar broad distribution pattern as that obtained from dextran filled lysosomes (fig.1.21). Dextran did not much affect the integrity of the cells. It slightly decreased the cell number, and slightly increased the cell volume, but did not much alter the amount of ouabain binding, (table.1.d). Cells treated with dextran showed

more fragility and sensitivity to shear than control cells. 10 Dounce homogenizer strokes were able to produce a homogenate similar to that obtained by using 40 strokes in normal cells as determined by microscopic examination.

Fig.1.21 : B-hexosaminidase distribution pattern of dextran treated cells.

Cells were grown for 48 hours in the presence of 2% dextran-500 and when the particulate fraction was layered on the gradient, B-hexosaminidase (closed circles) distributed broadly throughout the gradient showing a peak of activity similar to that obtained from control cells, (open circles). Dextran treated cells loaded with ouabain and returned to growth medium containing 2% dextran for 24 hours found to have the same broad distribution (triangles). The results are the mean of three gradients.

(fig.1.21)


CUSHION
SUCROSE
39-40%
35-36%
30-31%
OVERLAY
ACTIVITY
PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

(table 1.d)

	cell number X1E-06Cell per plate	cell volume Cubic u/cell	ouabain binding molecules/cell X1E-03
control	1.21 ±0.2	1713 ± 63	740 ± 62
Dextran treated	0.78 ±0.9	2144 ± 68	877 ± 115

Table 1.d : The effect of dextran on HeLa cell number, cell volume and ouabain binding.

Part II

The effect of chloroquine on cardiac glycosides accumulation and release, and on lysosomal enzyme activity in HeLa cells

Methods

Cell culture :-

HeLa cells were grown either on Roux bottles as described in part I or on 5 cm^2. Nunc petri dishes with growing surface area of 20 cm^2. as follows :-

0.2 X1E+06 cells were seeded on plates in 4 ml (BME) growth media supplemented with 10% v/v heat inactivated newborn calf serum, 0.281 mg/ml L-glutamine, and 100 units/ml penicillin-streptomycin. Cultures were gassed with mixture of 5% CO2 and 95% air after they were placed in a plastic box, tightly sealed, and grown to confluency at 37 C.

Isolation and determination of phospholipids:-

Total lipids were extracted from the particulate fraction prepared as outlined in part I, by the method of Folch et al. (1957). Total lipid phosphorus was determined by the method of Rouser et al. (1969).

Assays:-

Protein :-

As described in part I.

Enzymes assayes :-

As described in part I.

chloroquine treatment :-

Determination of cardiac glycosides accumulation :-
Cells were grown to confluency on Roux bottles with a concentration of 1 X1E-04 M chloroquine added to the media in the presence of cardiac glycoside (1X1E-08 M) as described under chronic treatment in part I.

Determination of cardiac glycoside release:-

Cardiac glycoside pulsing was carried out as described in part I, except that cells were returned after pulsing to normal growth media which contains 1 X1E-04 M chloroquine.

Determination of enzymes activity :-

B-hexosaminidase and lactate dehydrogenase activity:-

Cells were grown on 5cm^2 plates to confluency and then media was replaced with fresh media contains 100, 200, or 500 uM chloroquine and cultures were incubated for 5 hours at 37° C. After incubation media and cells were collected separately and assayed for B-hexosaminidase and lactate dehydrogenase activity as described in part I. The cells

were collected by scraping.

The effect of chloroquine on phospholipids :=

Cells were grown on Roux bottles for 48 hours as described in part I, in (BME) growth medium containing 10,50,or100 uM chloroquine. Cells were then collected, washed and lipids were extracted. Phospholipids were determined as described in isolation and determination of phospholipids.

Materials

chloroquine and ascorbic acid (vitamin C) were obtained from sigma chemical company, ammonium chloride was obtained from BDH chemical ltd. (poole, England). All other materials were outlined in part I.

Results

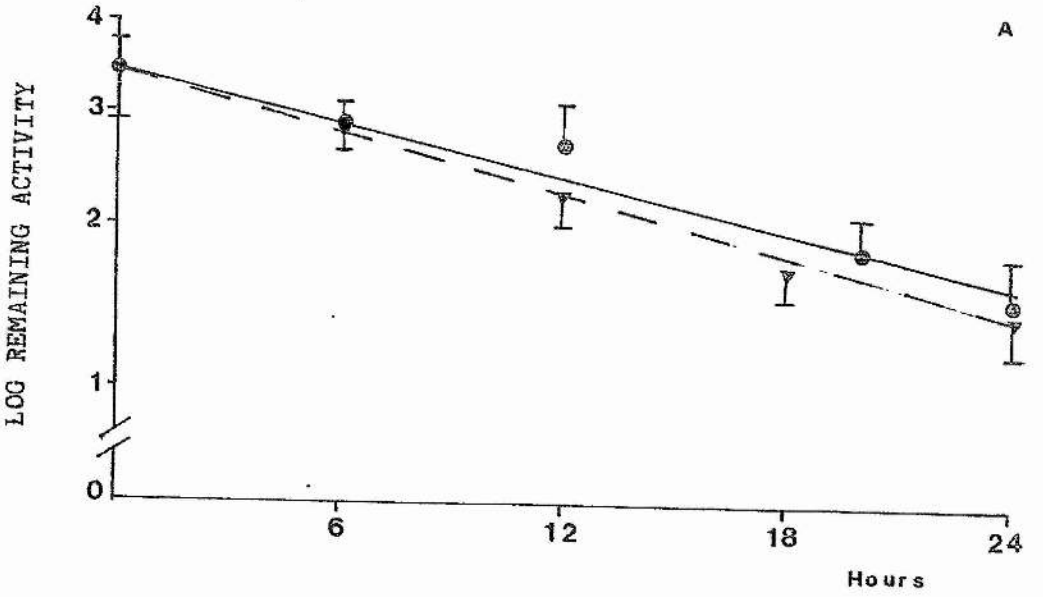
Effect of chloroquine on the release and accumulation of ouabain:-

HeLa cells were pulse labelled with 2X1E-07 M ouabain and returned to normal growth medium +1X1E-04 M chloroquine. The slow rate of excretion has not been affected by chloroquine (fig.2.1,a) and both control and chloroquine treated cells showed similar distribution patterns at 0 time and 24 hours after loading (fig.2.2). Cells treated chronically with ouabain in the presence of 1X1E-04 M chloroquine showed a slight decrease in the amount of accumulation (fig.2.3,a). On the sucrose gradient, accumulated ouabain showed two peaks of activity. One at 30-31% w/w sucrose, the 5'-nucleotidase peak fraction, and the second at 35-36% w/w sucrose similar to that of the B-hexosaminidase. Also a substantial amount of activity was found in the top of the gradient (fig.2.4). The distribution pattern in chronically treated cells seems to be composed of a combination of two patterns , the 0 time and 24 hours patterns obtained from ouabain loaded cells. Chloroquine was found to have no significant effect on the distribution pattern of accumulated ouabain apart from slight decrease in the lysosomal peak, (fig.2.4).

Fig.2.1,a&b : The effect of chloroquine on the excretion rate of ouabain and digoxin.

Cells were loaded with 2X1E-07 M ouabain or digoxin in k free krebs, washed for 15 min, and returned to growth medium + 1X1E-04 M chloroquine. At each time point cells were analysed for radioactivity and proteins. The log of remaining activity per mg protein was plotted against time and the best fit line (by eye) was drawn. Fig.a shows the excretion rate of ouabain (closed circles) with a t1/2 = 22 hours. Chloroquine (triangles) did not much alter this rate. Fig.b shows that digoxin (closed circles) was lost with a faster rate (t1/2 = 6.5 hours), and chloroquine (triangles) greatly slow the rate of excretion (t1/2 = 9 hours). The results are the mean of 3-5 experiments for each point ± S.D.

(fig.2.1.a&b)


A
LOG REMAINING ACTIVITY
4
3
2
1
0
6
12
18
24
Hours

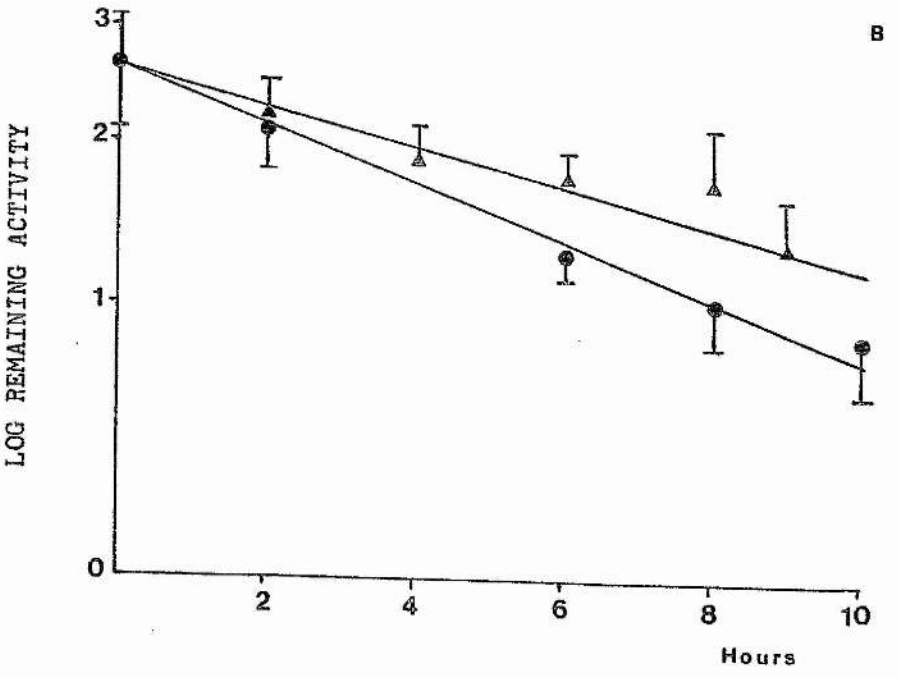

B
LOG REMAINING ACTIVITY
3
2
1
0
2
4
6
8
10
Hours

Fig.2.2 : The effect of chloroquine on the release of ouabain.

Cells were loaded with ouabain and the particulate fraction was prepared immediately after binding (squares) or 24 hours later (closed circles), or 24 hours later in the presence of 1X1E-04 M chloroquine (.....). Proteins were detected in the particulate fraction then layered on the gradient. The amount of ouabain in each gradient fraction was detected and expressed as n mols / mg layered protein. chloroquine did not affect the release of ouabain or its compartmentation. Arrows shows different markers peak activity. (MIT) succinate dehydrogenase, (LYS) B-hexosaminidase, (PM) 5'-nucleotidase, and (Y) lactate dehydrogenase. The results are the mean of three gradients. Number of experiments = 9.

(fig.2.2)

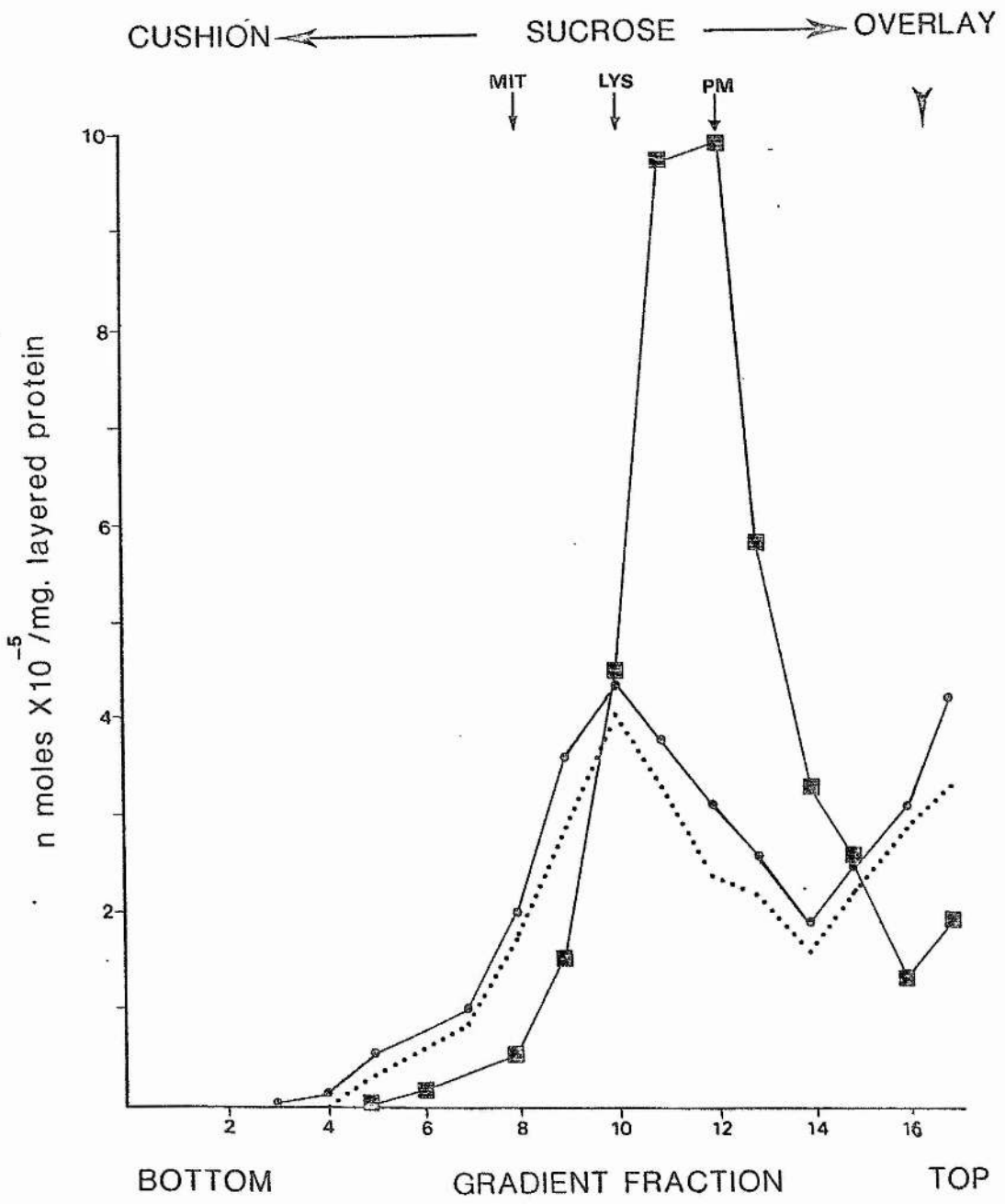

CUSHION
SUCROSE
OVERLAY
MIT
LYS
PM
n moles X10 -5 /mg. layered protein
10
8
6
4
2
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.2.3,a&b : The effect of chloroquine on the accumulation of ouabain and digoxin by whole cell.

Cells were grown for 48 hours in growth medium containing 1X1E-08 M ouabain or digoxin ± 1X1E-04 M chloroquine. Fig.a shows that chloroquine slightly decreased the accumulation of ouabain (about 19% decrease). While greatly increased (43% increase), that of digoxin as shown in fig.b. Dotted columns are chloroquine treated, and clear columns are controls . The results are the mean of four experiments ± S.D.

(fig.2.3.a&b)

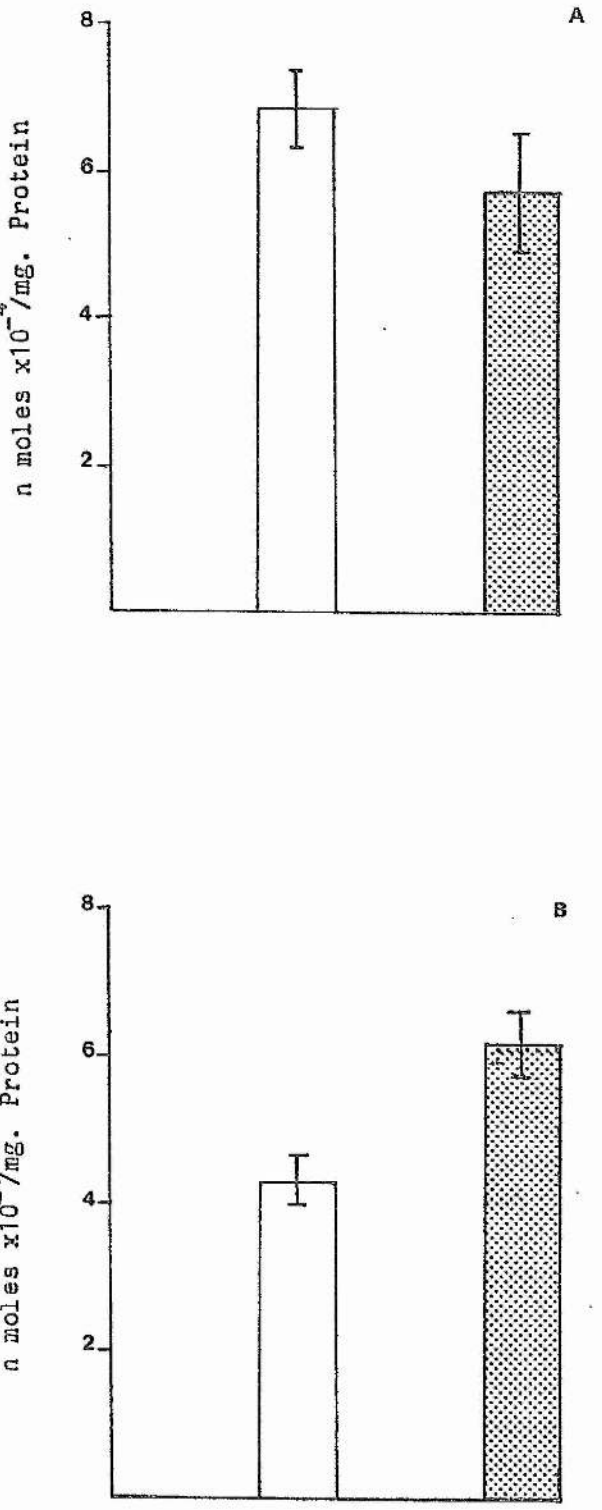
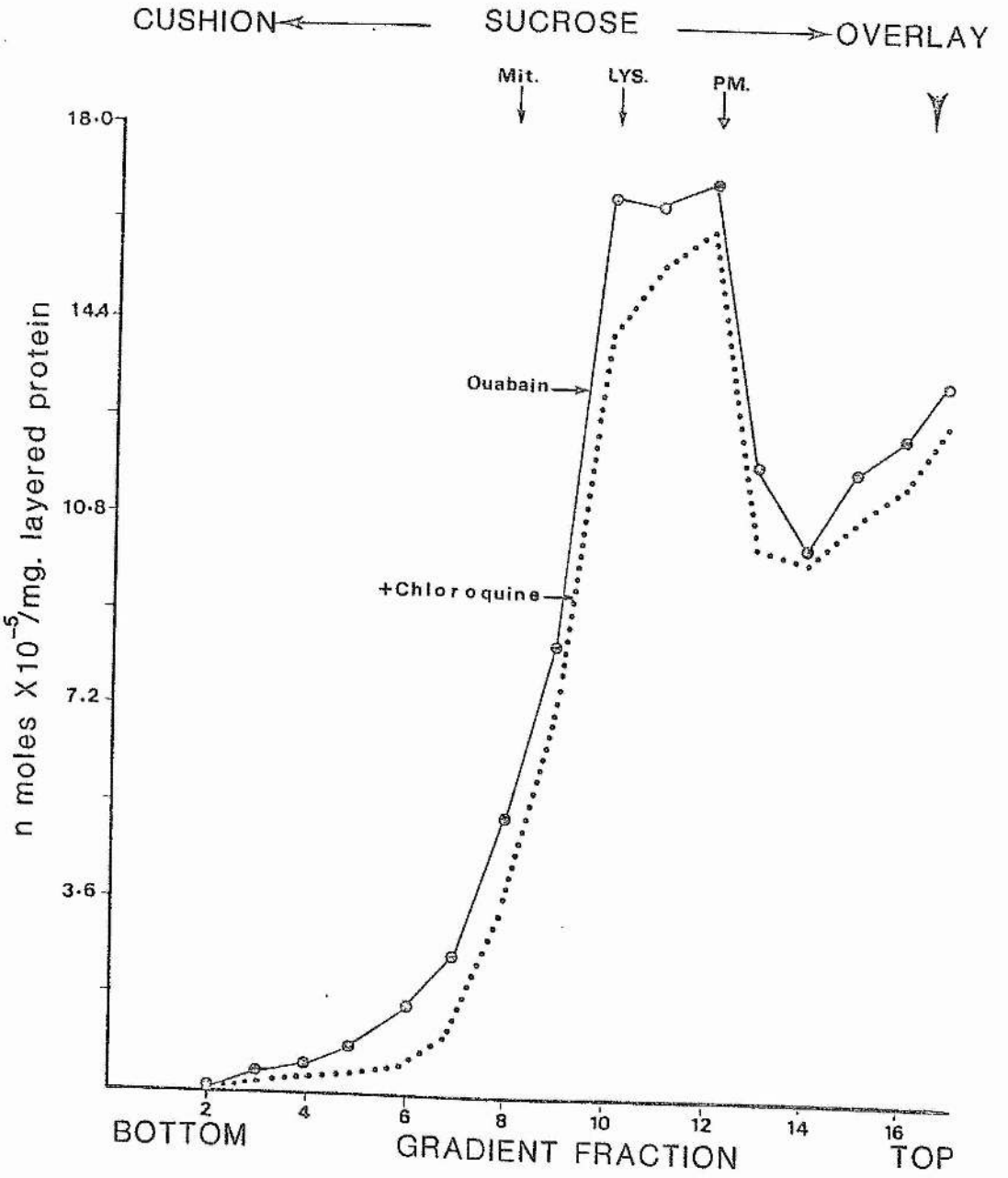


Fig.2.4 : The effect of chloroquine on ouabain accumulation.

Cells were grown for 48 hours in growth medium containing 1X1E-08 M ouabain + 1X1E-04 M chloroquine, rinsed, and the particulate fraction was prepared. Proteins were estimated in the particulate fraction, and then layered on the sucrose gradient. The activity of ouabain in each gradient fraction was determined and plotted as activity per mg layered protein. The distribution pattern of chloroquine untreated cells (⊖——⊖), and that of chloroquine treated cells (.....) were not much different, chloroquine slightly decreased the lysosomal peak but did not much alter the distribution. The results are the mean of three gradients.

(fig.2.4)


CUSHION
SUCROSE
OVERLAY
Mit.
LYS.
PM.
18·0
14.4
10·8
7.2
3·6
n moles X10⁻⁵/mg. layered protein
Ouabain
+Chloroquine
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Effect of chloroquine on digoxin release and accumulation:-

Chloroquine greatly slows the excretion rate of digoxin (fig.2.1,b), this is thought to be through the effect of chloroquine on the internalization pathway or at the lysosomal stage. The distribution pattern of internalized digoxin showed that retained activity of digoxin was increased in the presence of chloroquine giving an increase in the lysosomal peak (fig.2.5). On the other hand the great accumulation of digoxin in chronically treated cells (fig.2.3,b), caused by chloroquine was mainly due to its effect on the lysosomal compartment by retaining more molecules in the lysosomes as shown in (fig.2.6). Digoxin in chloroquine untreated cells showed two peaks throughout the gradient, one similar to the plasma membrane marker which resembles the surface bound activity and a second peak matching the lysosomal marker peak fraction, which represents the accumulated digoxin molecules. But the top of the gradient was still clear of any significant soluble activity (fig2.6).

Effect of chloroquine on B-hexosaminidase distribution, activity and release:-

Although chloroquine did not affect the lysosomal buoyant density (fig.2.7), causing no shift in its distribution pattern throughout the sucrose gradient, it greatly inhibited B-hexosaminidase activity when cells were exposed to different concentrations of chloroquine (fig.2.8,a), in a dose independent manner.

Ngaha and Akanji, (1982); and Jessup, et al., (1982) showed that chloroquine has the ability of inducing release of lysosomal enzymes into the outer medium of rat kidney lysosomes and human peripheral blood monocytes. Chloroquine was found to have no such effect on HeLa cells when B-hexosaminidase activity was determined in the growth medium after 5 hours of exposure to high concentration of chloroquine, (fig.2.8,a). Nor lytic activity was found at these concentration as determined by measuring the lactate dehydrogenase activity in both the cultures and the growth medium (fig.2.8,b). This result indicates that the minor decrease in the accumulation of ouabain caused by long exposure of the cells to chloroquine was not due to the possible release of ouabain trapped in the lysosomes, nor it was caused by leaking out of the lysed cells.

Effect of chloroquine on phospholipids:-

Chloroquine among other weak bases inhibits lysosomal phospholipases A and C, (Matsuzawa and Hostetler, 1979); and (Hostetler and Richman, 1982). As digoxin Known to have lipophilic property, unlike ouabain, we have studied the effect of chloroquine on phospholipids. The particulate fraction prepared from cells grown for 48 hours in the presence of different concentrations of chloroquine, showed great amount of phospholipids accumulation when concentration as small as 10 uM was used, (table 2.a). This result gave an indication on the possible relation between the accumulation of digoxin and phospholipids caused by chloroquine.

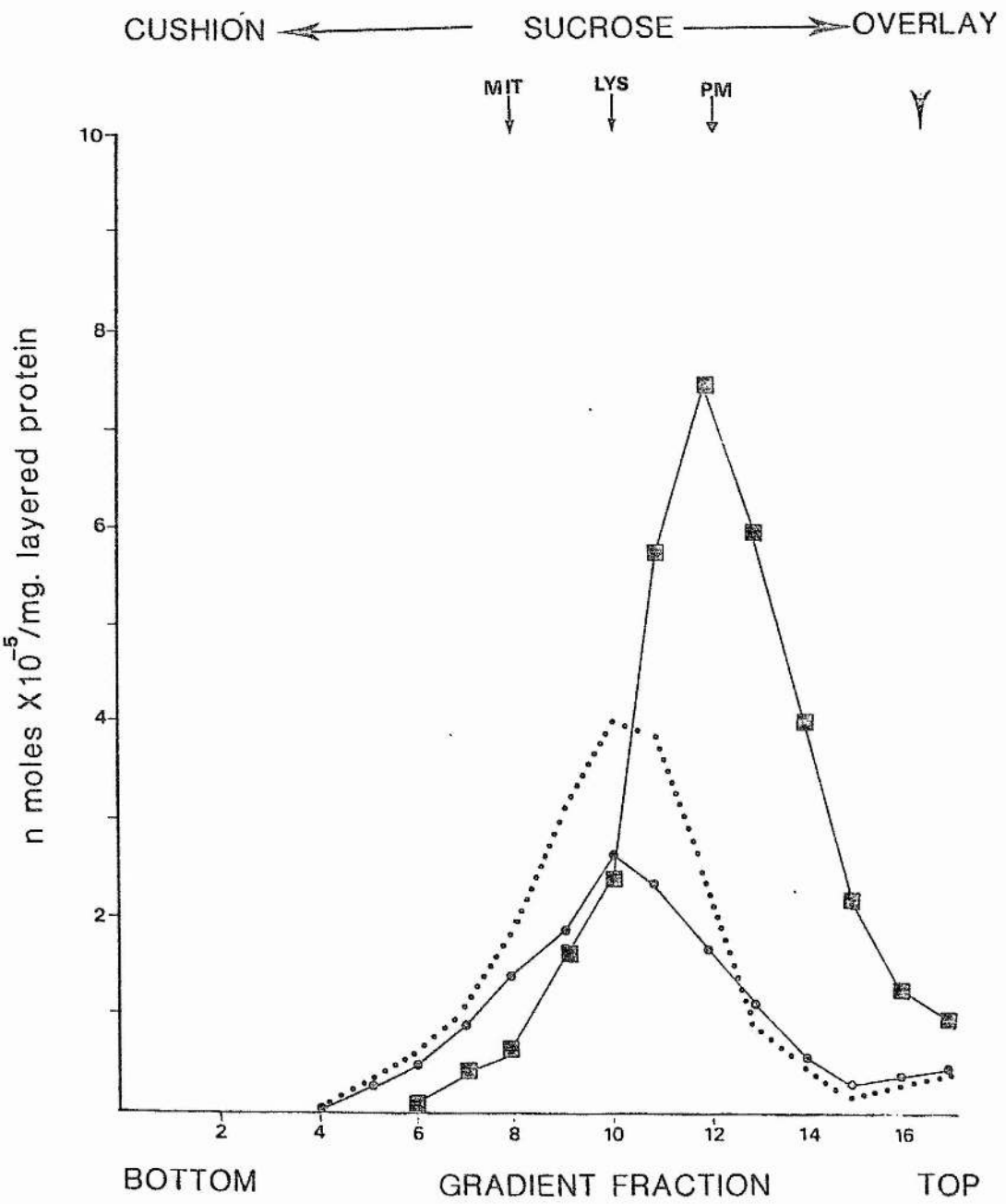
The effect of ammonium chloride on ouabain release:-

This weak base was found to have no effect, same as chloroquine on the release of ouabain from HeLa cells loaded with ouabain and returned to growth medium containing 1X1E-02 M ammonium chloride (fig.2.9).

Fig.2.5 : The effect of chloroquine on the excretion of digoxin.

Cells were loaded with digoxin, washed and returned to normal growth medium + 1X1E-04 M chloroquine for up to 9 hours. chloroquine treated cells retained more digoxin 9 hours after pulse labaelling(.....), than untreated cells (•——•). The retained activity caused by chloroquine showed a peak of activity similar to that of the lysosomal marker, but different from that obtained immediately after binding (squares). symbols and conditions are similar to that of fig.2.2.

(fig.2.5)


CUSHION
SUCROSE
OVERLAY
MIT
LYS
PM
n moles X10⁻⁵/mg. layered protein
10
8
6
4
2
2
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6
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10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.2.6 : The effect of chloroquine on digoxin accumulation.

Cells were grown in the presence of 1X1E-08 M digoxin + 1X1E-04 M chloroquine for 48 hours. The distribution pattern of the particulate fraction prepared from chloroquine untreated cells (•——•), showed two peaks of activity, in position similar to that of the lysosomal marker and the plasma membrane marker. Very little soluble activity was found in the overlay. Chloroquine treated cells (....) showed only one sharp peak at the lysosomal peak fraction with great increase in the accumulated amount of digoxin. symbols and conditions are similar to that of fig.2.4.

(fig.2.6)

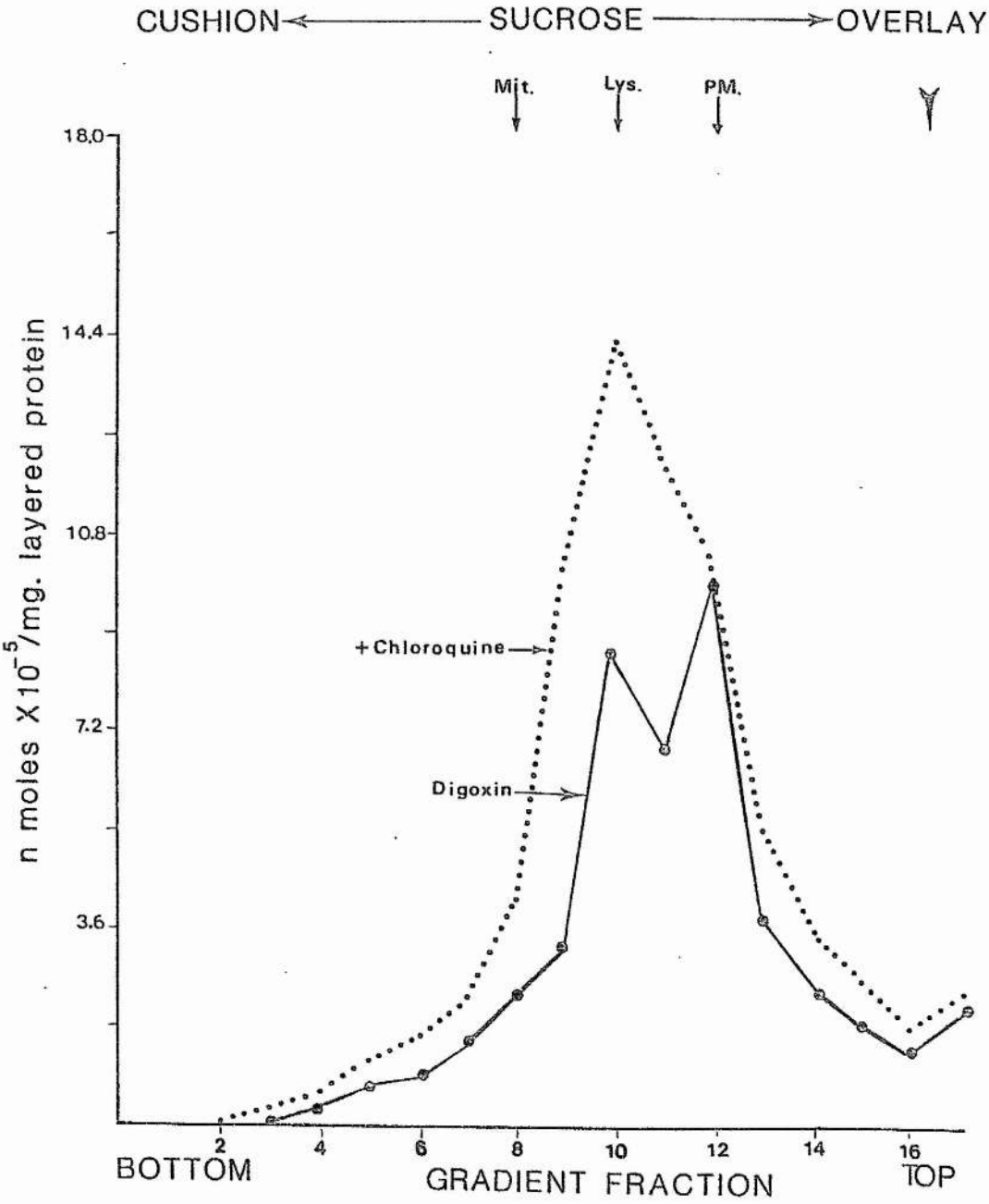

CUSHION
SUCROSE
OVERLAY
Mit.
Lys.
PM.
18.0
14.4
10.8
7.2
3.6
n moles X10⁻⁵/mg. layered protein
+Chloroquine
Digoxin
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Fig.2.7 : The effect of chloroquine on the distribution of B-hexosaminidase throughout the linear sucrose gradient.

The distribution patterns of B-hexosaminidase activity in particulate fractions prepared from control and chloroquine treated cells were detected throughout the sucrose gradient. Both control (——) and chloroquine treated (-·-·-) distributions were identical. The results are plotted as the activity of each fraction expressed as the percent of peak activity fraction. The results are the mean of three gradients.

(fig.2.7)

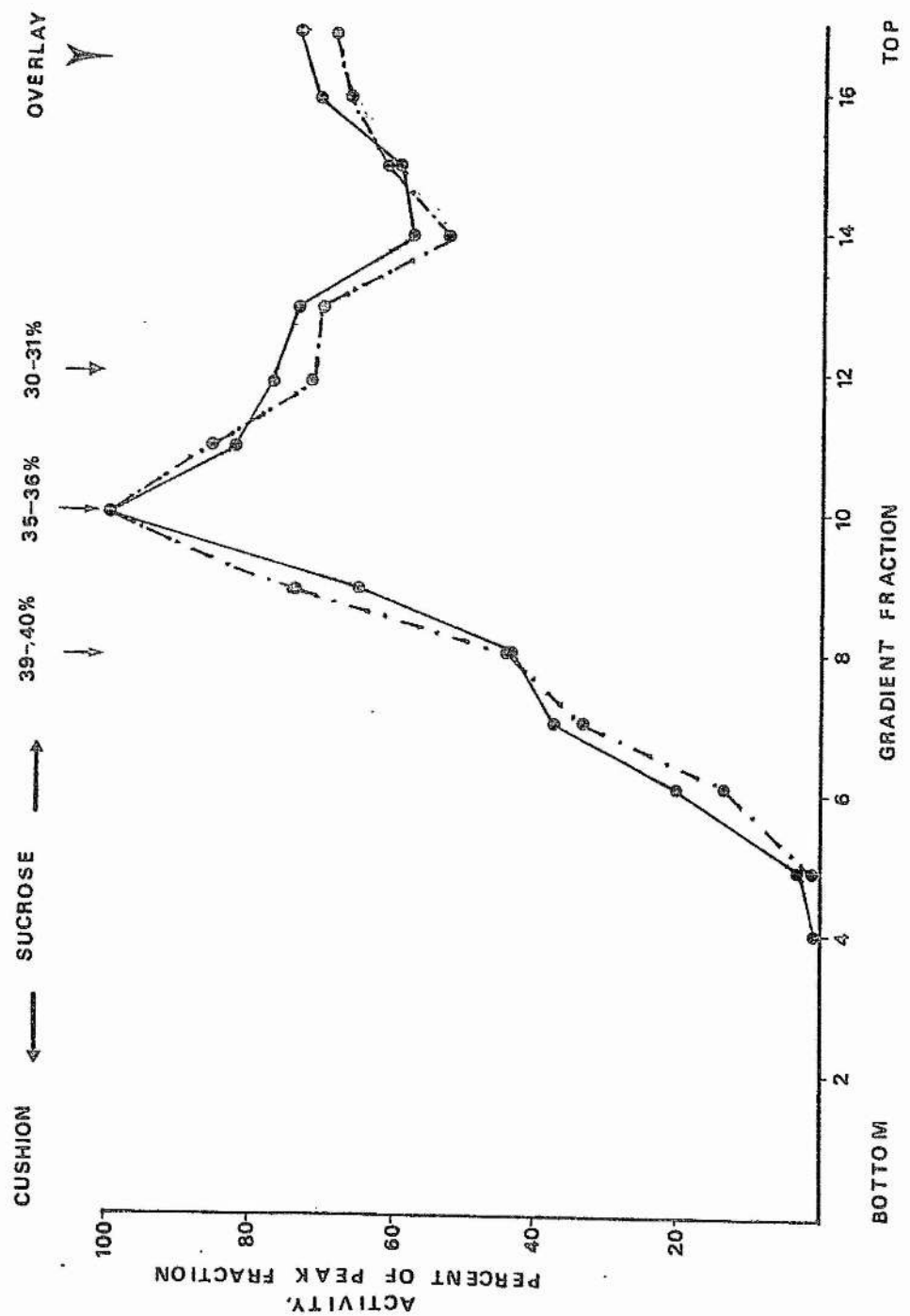
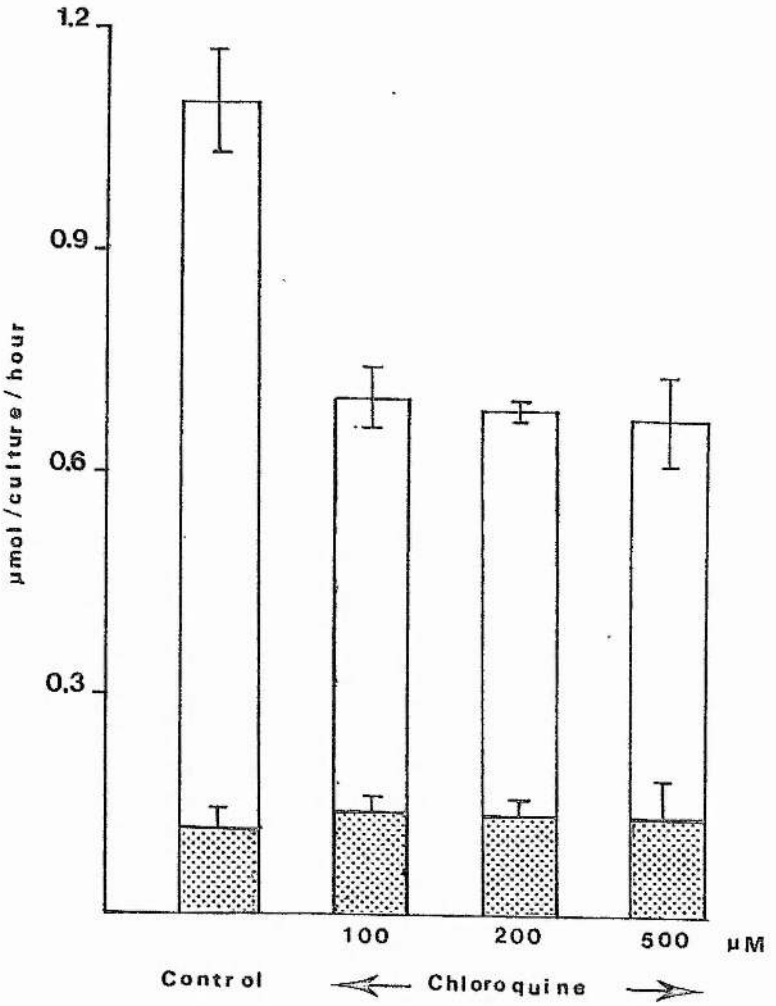

OVERLAY
30-31%
35-36%
39-40%
SUCROSE
CUSHION
ACTIVITY, PERCENT OF PEAK FRACTION
100
80
60
40
20
BOTTOM
2
4
6
8
10
12
14
16
TOP
GRADIENT FRACTION

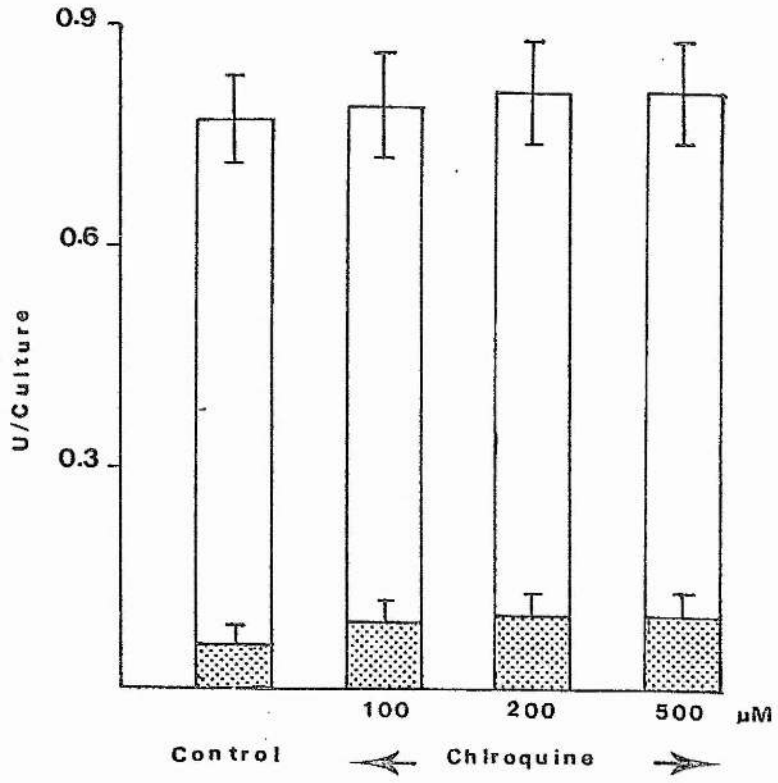
Fig.2.8,a&b : The effect of chloroquine on B-hexosaminidase and lactate dehydrogenase activities and release.

cells were grown on plates for four days. Then exposed to different concentrations of chloroquine for 5 hours. The culture (plane columns) and the medium (dotted columns) were assayed separatly for B-Hexosaminidase (fig.a) and lactate dehydrogenase (fig.b) activities. Chloroquine greatly decreased B-hexosaminidase activity, but did not alter it's release. and has no lytic activity on Hela cells in concentrations as high as 500 uM. Results are the mean of three experiments expressed as enzyme activity / culture / hour ± S.D. (4 plates per experiment).

(fig.2.8,a)


1.2
0.9
0.6
0.3
µmol / culture / hour
100
200
500
µM
Control
Chloroquine

(fig.2.8,b)



(table 2.a)

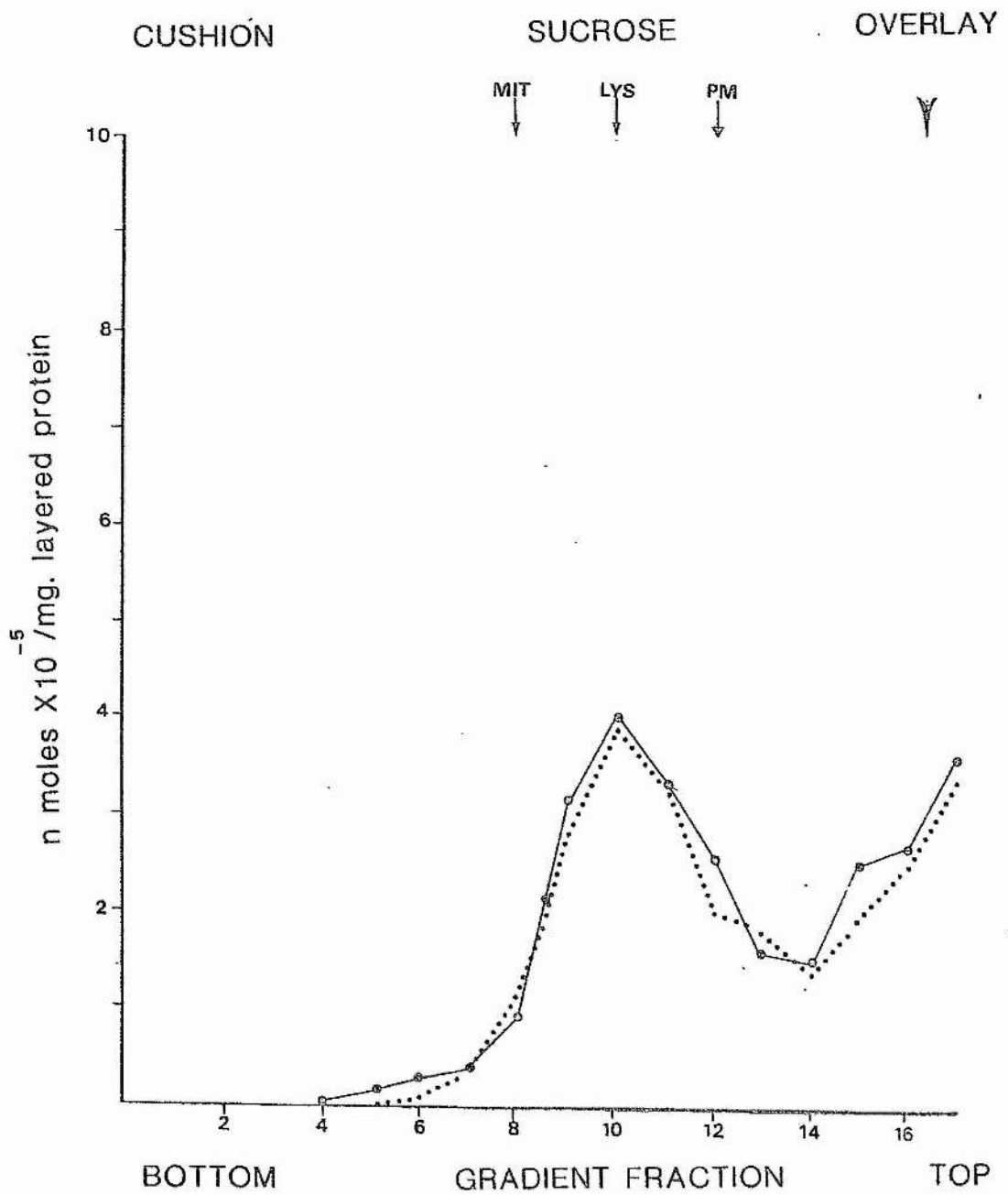
	ug phosphorus/mg protein
control	0.275 ± 0.083
10 uM chloroquine	0.511 ± 0.071
100 uM chloroquine	0.663 ± 0.067

Table 2.a : the effect of chloroquine on the accumulation of phospholipids in HeLa cells.

Fig.2.9 : The effect of ammonium chloride on ouabain release.

Cells were loaded with ouabain for 20 min, washed, and returned to growth medium + 1X1E-02 M ammonium chloride for 24 hours. Ammonium chloride showed no effect on ouabain release. (——) controls, and (.....) drug treated. conditions and symbols are similar to that of fig.2.2.

(fig.2.9)


CUSHION
SUCROSE
OVERLAY
MIT
LYS
PM
n moles X10 -5 /mg. layered protein
10
8
6
4
2
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

Part III.

The effect of low temperature, cells grown in suspension, vinblastine and nocodazol on HeLa cells handling of cardiac clycosides after binding to the cell surface

Methods

Cell cultures :-

Cells were grown on Roux bottles or $5cm^2$ plates supplemented with 10% v/v new born calf serum, 0.281 mg/ml L- glutamine, and 100 units/ml penicillin-streptomycin as described in part I, and II.

Determination of ouabain release and distribution pattern in cells grown in suspension:-

Cells were grown to confluency on Roux bottles, pulsed with [3H] ouabain as described in part I, washed with 5mM K^+ Krebs, trypsinized, diluted with 20 ml growth media, dispersed using 5 passages through stainless steel needle by sterile plastic syringe, and suspended in 200 ml (BME) growth media. Supplemented with 10% v/v newborn calf serum, 0.281 mg/ml L-glutamine, and 100 units/ml penicillin-streptomycin and stirred at 140 revolution/min. in 250 ml rubber stoppered "Bellco" spinner flask at 37° C. Samples were taken at 12 and 24 hours ,centrifuged at 2,000 rpm. for 5 min, in (MSE) coolspin centrifuge to sediment the cells , and washed with cold 5mM K Krebs contains 1% v/v newborn calf serum. Aliquots were taken for [3H] ouabain radioactive determination, and for protein assay. The remaining cells were homogenized, the particulate fraction was prepared, layered on linear sucrose gradient, and the

distribution pattern was detected as described in part I.

Determination of B-hexosaminidase activity of cells grown in suspension :-

Cells grown for four days on Roux bottles were left to grow in suspension for 24 hours as described above. After incubation a sample of cells was sediment, washed, resuspended in homogenizing buffer, and assayed for B-hexosaminidase activity as described in part I. The result was compared with that of control cells grown on Roux bottles only and collected either by scraping off glass with small brush or by adding 5ml 0.25% trypsin solution in earle's balanced salt (magnesium and calcium free), for 20 min, at 37° C. After the cells were detached trypsin was neutralised with 20 ml of serum free (BME) media, centrifuged at 2,000 rpm. for 5 min, as described before, washed with 10 ml Earle's balanced salt and resuspended in homogenizing buffer, and assayed for B-hexosaminidase activity as described in part I.

Effect of low temperature :-

Cells were grown on Roux bottles to confluency and then labelled with [3H] ouabain as described in part I. The bound activity at zero time was determined, other cultures were returned to normal growth media and incubated at 2° C on ice in a refrigerator for 24 hours, the ice being changed

every 12 hours. samples from 0, and 24 hours experiments were washed and the bound activity/mg protein was determined as described in part I. The remaining samples were fractioned, layered on sucrose gradient, and distribution patterns were detected as outlined in part I.

Determination of ouabain and digoxin release in cells treated with vinblastine or nocodazol:-

Cells were grown for four days in 5 cm^2 plastic petri dishes then media was decanted, cells were rinsed with K free Krebs , and pulsed with ouabain as described before. Cells were then returned to (BME) growth media ± 5X1E-05 M vinblastine or 10µg/ml nocodazol for up to 24 hours. At various times between 0-24 hours, the media was decanted, the plates rinsed with cold 5 mM K^+ Krebs, and trypsinized for 15 min, The trypsin was neutralized with serum, the cells suspended as usual, and the cell number and volume were estimated in the coulter counter. [3H] ouabain was measured as usual. The results were calculated as molecules /cell 1X1E-03. Cells loaded with digoxin were returned to normal growth medium for up to 8 hours and the remaining activity at each time point was determined as described above.

Materials

Vinblastine (sulphate salt), and methyl[5-(2-thienyl carbonyl)-1H -benzimidazol-2-YL] carbamate, (nocodazol), were obtained from Sigma chemical company. All other materials were outlined in part I.

Results

The effect of cells grown in suspension on lysosomal activity and ouabain handling by HeLa cells:-

HeLa cells grown for four days on Roux bottles, trypsinized and left to grow in suspension for up to 24 hours showed great decrease in B-hexosaminidase activity, (table 3.a). This reduction in lysosomal enzyme activity was not due to the effect of trypsin because cells treated with trypsin and immediately assayed for B-hexosaminidase activity showed no change in the enzyme specific activity when compared with control cells, (table 3.a). The previous result indicates that variation in cell shape, caused by growing cells in suspension might result in different biochemical properties, (Folkman, and Moscona, 1978). But does this affect the lysosomal handling of internalized ouabain?. Cells which were left to grow in suspension did not retain ouabain 24 hours after being pulsed. The explanation was that growing ouabain loaded cells in suspension , did not prevent the internalization of the ligand to the lysosomal compartment, but enhanced its release (fig.3.1).

(table 3.a)

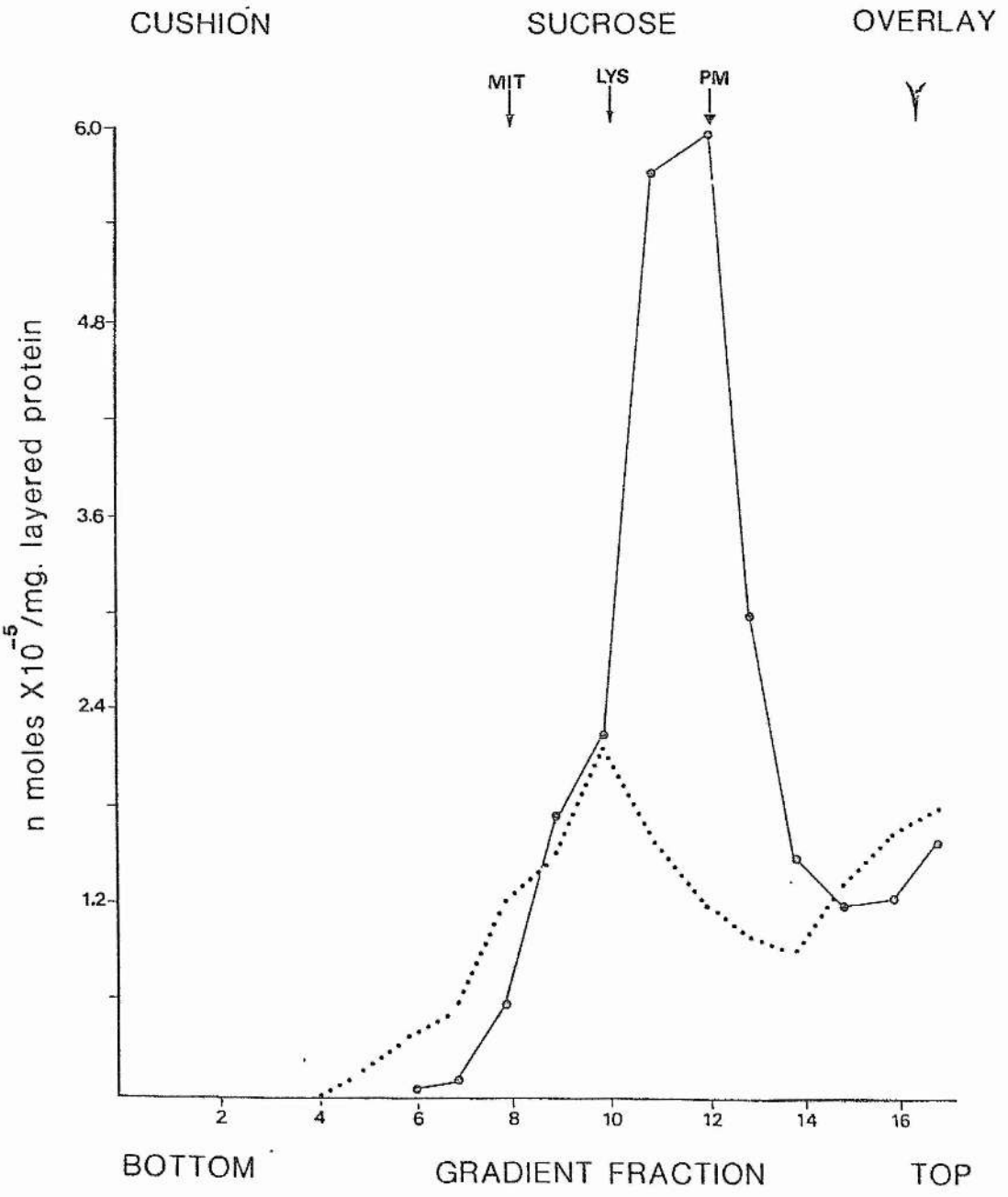
	scraped cells	trypsinized cells	cells grown in suspension
specific activity	0.203 ± 0.016	0.197 ± 0.024	0.110 ± 0.014

Table 3.a : The effect of cells grown in suspension on the activity of B-hexosaminidase. Cells were grown for four days and either collected by scraping or by trypsinization, and then assayed for enzyme activity or trypsinized and left to grow in suspension for 24 hours and then assayed. Both scraped and trypsinized cells showed the same specific activity. While cells grown in suspension showed great decrease in B-hexosaminidase activity. The results are the mean of three experiments ± S.D. calculated as u mol/mg protein/ hour.

Fig.3.1 : The effect of cells grown in suspension on the internalization and release of ouabain.

Cells were grown for four days, loaded with ouabain, and then left to grow in suspension for 24 hours. Immediately after binding, ouabain (•——•), co-distributed with 5'- nucleotidase (PM). 12 hours later (.....), ouabain was internalized and showed a distribution pattern similar to that of B-hexosaminidase (LYS). But 24 hours after growing cells in suspension the distribution pattern was undetectable as cells showed no retained activity at this time. The results are the mean of two gradients plotted as the activity in each fraction per mg layered protein.

(fig.3.1)


CUSHION
SUCROSE
OVERLAY
MIT
LYS
PM
6.0
4.8
3.6
2.4
1.2
n moles X10^{-5}/mg. layered protein
2
4
6
8
10
12
14
16
BOTTOM
GRADIENT FRACTION
TOP

The effect of low temperature:-

One of the main methods usually used to inhibit the internalization of a surface bound ligand is low temperature, (2° - 4°c), because internalization does not occur in chilled cells, (Carpenter, and Cohen 1976), (Wall, and Hubbard, 1981), and (Iacopetta, and Morgan, 1983).

If HeLa cells were left to grow in normal growth medium at 2 C for up to 24 hours after they have been pulsed with ouabain, 85 % of the total initial bound activity remained associated with the cell (fig.3.2,a) and the distribution pattern of the particulate fraction prepared from these cells showed that all the bound activity co-distributed with the plasma membrane marker, 5'-nucleotidase (fig.3.2,b). i,e. associated with the cell surface. This also shows that dissociation has a limited role in the excretion mechanism of ouabain bound to the sodium pump. Because less than 15% of the initial bound activity was lost at 2° C 24 hours after ouabain binding.

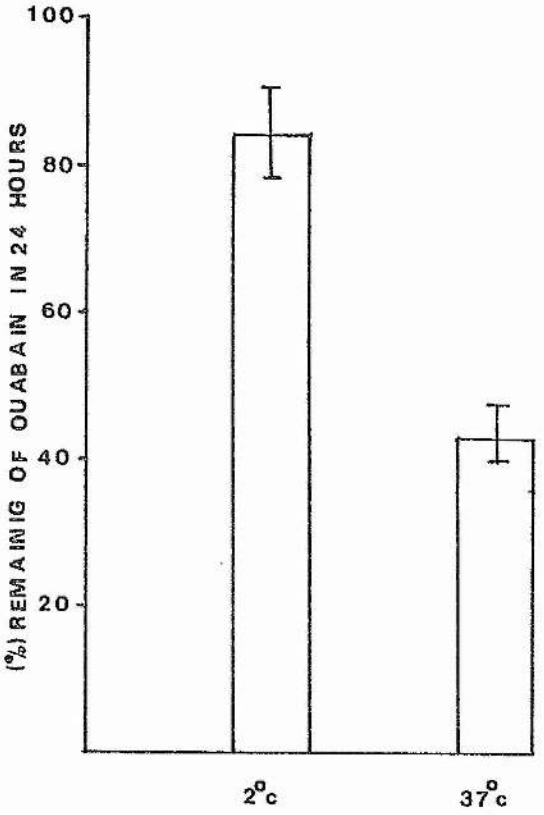
The effect of nocodazol and vinblastine:-

Vinblastine and nocodazol are among those agents which have the ability to disrupt the microtubules structure within the cell, (Deysson, 1975; and de Brabander et al., 1975). Therefore, if those agents were applied to the cells, it was expected that they might interfere with the

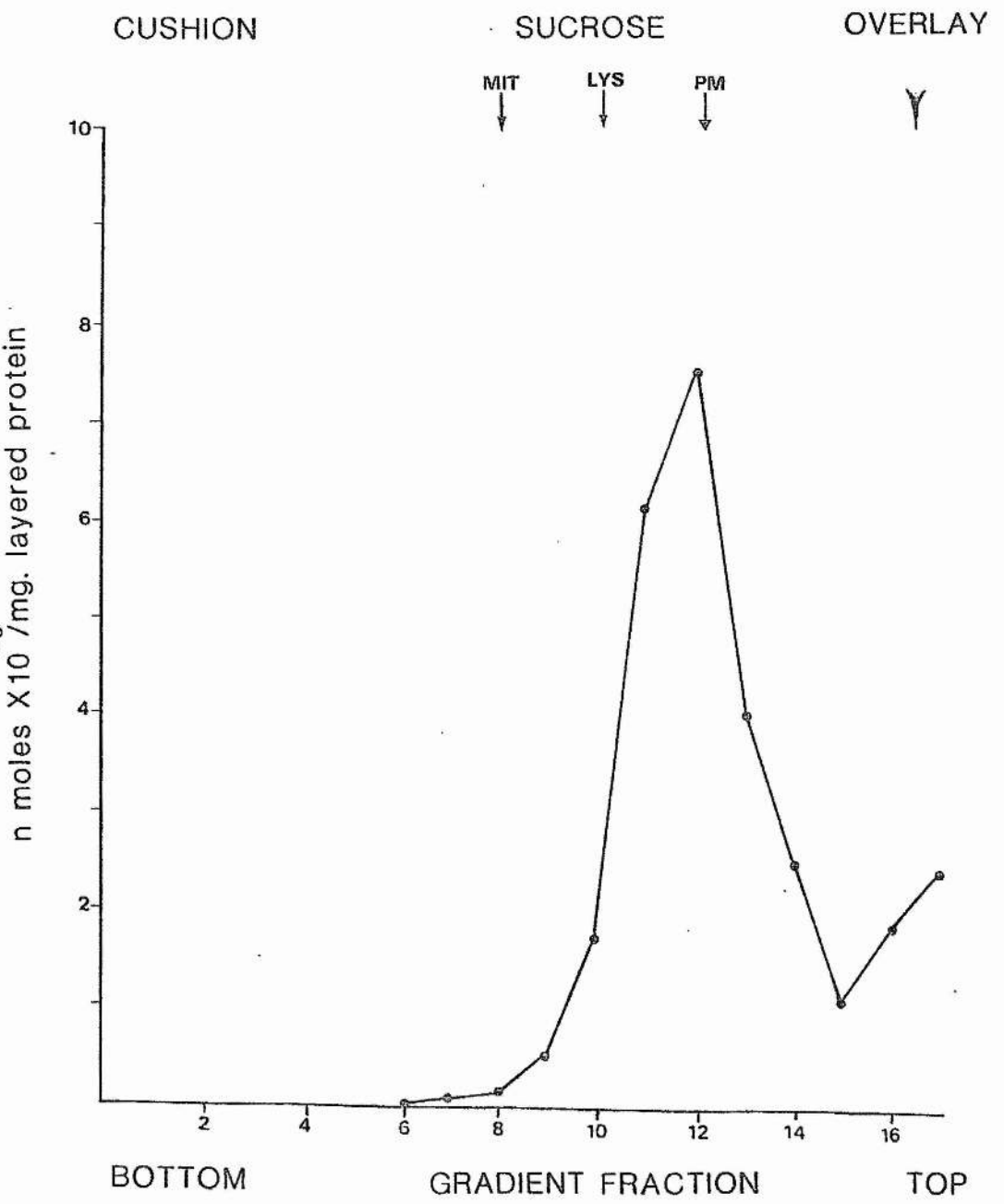
Fig.3.2,a&b : The effect of low temperature on the handling of ouabain after binding to Hela cell surface.

Cells were loaded with 2X1E-07 M ouabain in K free krebs at 37c for 20 min, washed, and immediately chilled by incubation at 2 c in normal growth medium for 24 hours. Fig.a shows that 85% of the initial bound activity still associated with the cell 24 hours after loading cells with ouabain. Fig.b shows that the associated activity was not internalized and still associated with the cell surface. The results are the mean of three experiments ± S.D.

(fig.3.2,a)



(fig.3.2,b)


CUSHION
SUCROSE
OVERLAY
MIT
LYS
PM
n moles X10 -5 /mg. layered protein
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BOTTOM
GRADIENT FRACTION
TOP

excretion rate of internalized ouabain. However, when cells loaded with ouabain were returned to growth medium containing 5X1E-05 M vinblastine or 10 μg/ml nocodazol, for up to 24 hours. Both were found to have no detectable effect on the excretion rate of ouabain (fig.3.3), while cells loaded with digoxin then returned to growth medium which contains 5X1E-05 M vinblastine excreted digoxin in a slower rate than that of control cells (fig.3.4)

Fig.3.3 : The effect of vinblastine and nocodazol on ouabain release.

Cells were loaded with [3H] ouabain for 20 min, in K free krebs containing 2X1E-07 M, washed for 15 min, at 37c in 5mM K krebs, and returned to growth medium ± 5X1E-05 M vinblastine or 10 ug/ml nocodazol. At each time point plates were analysed for remaining ouabain and cell number. The best fit line between 0 - 24 hours using Glim package gave the same slopes and intercepts for control (closed circles), vinblastine treated (open circles), and nocodazol treated (triangles). The rate of release estimated was (2.6±0.7 % per hour, 2.55±0.8 % per hour, and 2.8±0.36 % per hour) for controls, vinblastine treated, and nocodazol treated respectively. The results are expressed as log molecules per cell 1X1E-03. Points shown are the experimental coinciding (1-3) points.

(fig.3.3)

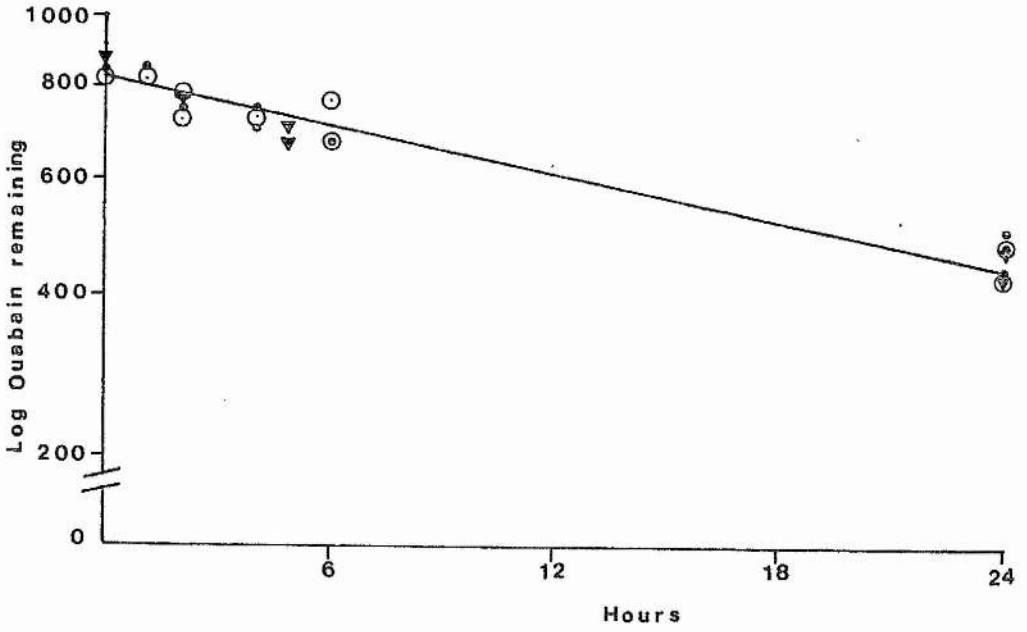
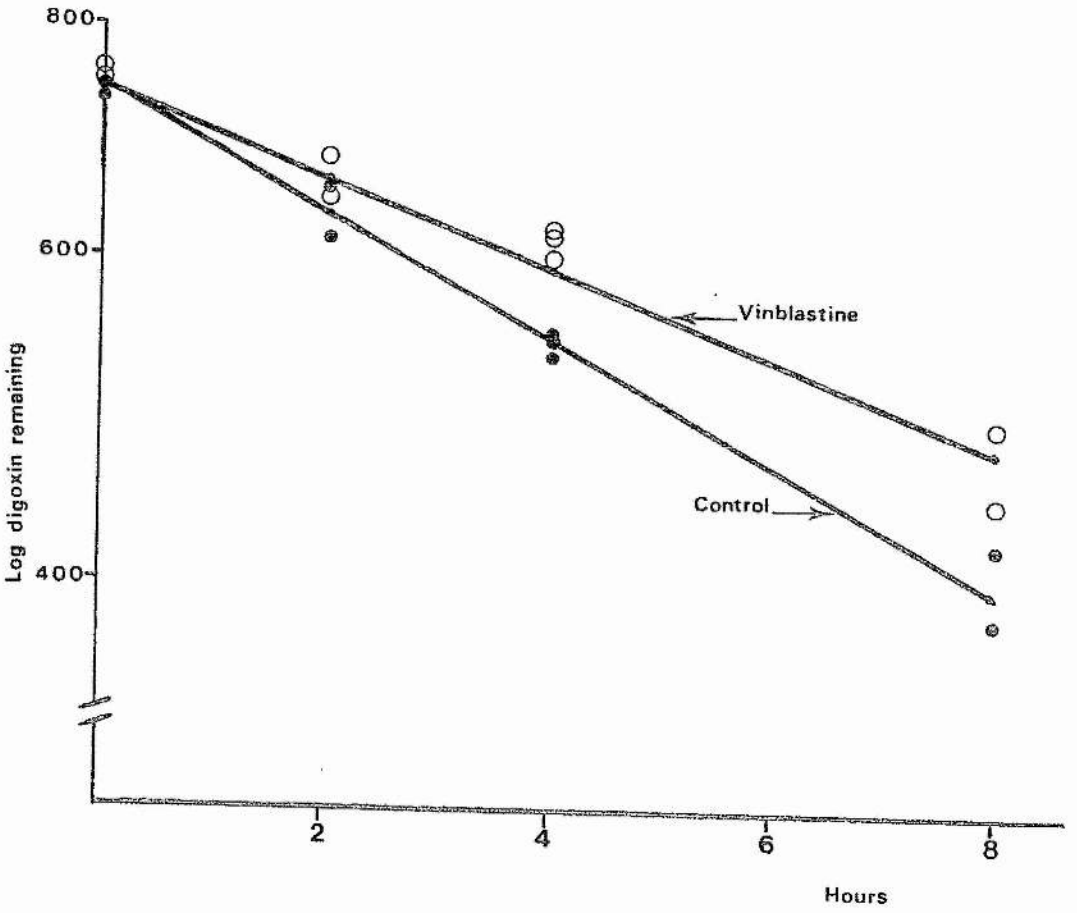


Fig.3.4: The effect of vinblastine on digoxin release.

Cells were loaded with [H]digoxin for 20 min in K krebs containing 2X1E-07 M digoxin , washed for 15 min at 37c in 5mM K krebs, then returned to growth medium ± 5X1E-05 M vinblastine. At each time point plates were analysed for remaining digoxin and cell number. The best fit line between 0-8 hours using Glim package gave the same intercept but different slopes for control (closed circles) and vinblastine treated (open circles). The rate of release estimated was 7.78 ± 0.83% per hour and 5.76 ± 0.72% per hour for controls and vinblastine treated respectively. The results are expressed as log molecules per cell 1X1E-03. points shown are the experimental coinciding (1-3) points.

(fig. 3.4)


800
600
400
Log digoxin remaining
Vinblastine
Control
2
4
6
8
Hours

DISCUSSION

When the binding of a ligand to a specific receptor is irreversible or has a low dissociation rate, recovery can occur by turnover of the receptor. In the case of cardiac glycoside bound to the cell there is little direct evidence about the pathway of internalization of cardiac glycosides, nor the regulation mechanism of the removal of Na,K-ATPase from the cell surface in order to turnover. However Cook & Brake (1978); and Griffiths, Lamb & Ogden (1983) showed that the recovery of pumping ability of sodium pump blocked with ouabain in Hela cells is much faster than the rate of excretion of blocking ouabain, and there is a good evidence that the accumulation of cardiac glycosides by the cell is a result of the blocking action of the cardiac glycoside on the sodium pump. For example when radioactive glycoside blocks the pump it is internalized and then lost very slowly from the cell, but if the pump is protected using high potassium concentration or non-radioactive glycoside a much smaller uptake by the cell occurs, which is readily washed out from the cell and by cell fractionation we showed that it failed to appear in a subcellular compartment. Moreover Pollack et al (1981b), calculated the turnover rate of Na,K-ATPase and found that in each generation the cell synthesizes four sets of Na,K-ATPase and removes three from

net increase of one, and that internalization of surface bound ouabain has the same rate as the turnover of ^{13}C-labelled amino acid in the subunit of Na,K-ATPase. This led to the suggestion that the uptake of ouabain specifically bound to the sodium pump is a marker for the internalization of pump in HeLa cells despite that the fully active Na,K-ATPase has not yet been clearly detected in any intracellular compartment. Knowing that internalized cardiac glycosides are not metabolized by these cells (Cook & Brake, 1978; and Griffiths et al., 1983). This gave the possibility of following the ligand immediately after binding and its subsequent uptake using cell fractionation technique. The distribution patterns of the particulate fraction prepared from HeLa cells in the linear sucrose gradient showed that ouabain, digoxin, and digitoxin immediately after binding co-distribute with the plasma membrane marker 5'-nucleotidase as expected for a surface bound ligands, and very little activity of these cardiac glycosides can be found in a soluble fraction at the top of the gradient nor in the cytosolic fraction (S-III), which match the non sedimentable activity of this marker. Internalized ouabain co-distributed with the lysosomal marker B-hexosaminidase with a substantial activity in the soluble fraction at the top of the gradient, and a higher cytosolic activity similar to the non sedimentable activity of B-hexosaminidase, which indicates the compartmentation of

the ligand in a subcellular vesicle. Moreover the results suggest that internalized ouabain is trapped in the lysosomes, the evidence for this is that ouabain was released from its subcellular compartment by shearing force. Once the lysosomes are ruptured both B-hexosaminidase and radioactive ouabain are found free in the soluble fraction at the top of the gradient, Which indicates that both activities are depending on lysosomal integrity. Dextran filled lysosomes although did not show clear shift in there density , they distributed more broadly throughout the sucrose gradient. Also dextran minimized lysosomal integrity by increasing lysosomal size (Slater, 1969), rendering them more susceptible to shearing force which result in increasing the soluble activity of B-hexosaminidase in the overlay. Internalized ouabain in Dextran treated cells matched the broad distribution and showed a higher soluble activity in the overlay. These results are consistent with those described by Cook et al., (1982), who showed that internalized ouabain was released from its subcellular compartment not only by shearing force but also by osmotic shock, while ouabain bound to the cell surface was not. It seems that the lysosomal "trap" is responsible for the slow excretion of ouabain from the cell. This is supported by the experiment in which ouabain bound to HeLa cells which were grown in suspension after loading with radioactive ouabain, was released very quickly if

compared with cells grown in the normal way on roux bottles. These cells after growing for 24 hours in suspension showed large decrease in B-hexosaminidase specific activity which might indicates less lysosomal formation in these cells. The decrease in lysosomal population could be a result of decrease in lysosomal enzyme synthesis which is important for the recognition required for the lysosomal enzyme packaging into vesicles, as a result of their rounded shape. It is believed that the variation in cell shape could alter the biochemical property of the cell (Folkman and Moscona, 1978). Experimentally ouabain did not mainly dissociated from these cells, because cells grown in suspension did not prevent its internalization but largely enhanced its release, (Fig.3.1). This may also account for the result obtained by Baker and Willis, (1970), who found that HeLa cells which were trypsinized and grown for several days in suspension, when blocked with ouabain, lost it in a rate of minutes rather than hours.

The internalization process of ouabain is energy dependant. If cells were chilled immediately after binding 85% of ouabain initial activity were found associated with the cell surface 24 hours after binding. This indicates that dissociation from the sodium pump has very limited role in the excretion mechanism of surface bound ouabain from Hela cells. Cook and Brake, (1978) showed that if cells are

poisoned and their ATP inhibited by NaN and 2-deoxyglucose , internalization of ouabain was blocked. The previous results are good evidence for the idea that Na,K-ATPase turnover plays a significant role in the recovery of HeLa cells from ouabain which blocks the sodium pump.

On the other hand, it was thought that digoxin and digitoxin are handled similarly to ouabain, but recently Griffiths, Lamb, and Ogden (1983), showed that although the pumping ability of sodium pumps blocked with digoxin or digitoxin recovers at similar rate to that if ouabain was used in HeLa cell, their excretion rate from the cell was much faster than that of ouabain. The larger accumulation of ouabain compared to digoxin is expected on this difference in excretion rate. It seems that ouabain, digoxin and digitoxin are behaving similarly during binding and internalization, but differently thereafter. In the present work we found that digoxin and digitoxin co-distributed with the plasma membrane marker immediately after binding, but internalized digoxin appears to be in a subcellular compartment which is not sensitive to shear, and internalized digoxin sedimentability does not depend on lysosomal integrity. Although the distribution pattern of internalized digoxin did not mimic the whole distribution pattern of B-hexosaminidase, the fraction of peak activity was shifted from the plasma membrane marker peak fraction to

equilibrate at the same density of the lysosomal maker in the sucrose gradient. Since we did not establish any evidence for the distribution of internalized digoxin with any other major subcellular compartment or even free soluble activity in the cytosol, it is possible to suggest that internalized digoxin might associate with a macromolecule located in the lysosomal membrane, perhaps due to digoxin lipophilic property. An alternative is that digoxin does not reach the lysosomal compartment but is excreted from a pre lysosomal vesicles probably endosomes. The evidence for the latter idea is yet to be obtained by successful separation of prelysosomal vesicles which contain the free ligand.

The distribution patterns of cardiac glycosides activity throughout the linear sucrose gradient described here represents a surface bound or internalized activity since incubation of particulate fraction with ouabain or digoxin failed to show similar accumulation by the particulate fraction. It is quite clear from this experiment that the uptake into subcellular content seen in the distribution patterns can not be accounted for by simple physical interaction of cardiac glycosides, digoxin in particular, with subcellular elements, and does not depend on redistribution of soluble activity after homogenization.

The effect of chloroquine:-

The lysosomal function of degradation in cultured cells can be specifically inhibited by chloroquine (Lie and Schofield, 1973), and other weak bases (Matsuzawa and Hostetler, 1980). It is believed that they do so by elevating the intralysosomal pH. It is also believed that they interfere with the delivery of various ligands to the lysosomes either by inhibiting the internalization pathway (Sando et al., 1979; and Fitzgerald et al., 1980), or by inactivating the lysosomal process in receptor mediated endocytosis (Helenius et al., 1980). Griffiths et al., (1983) were the first to show that chloroquine, ammonium chloride and amantadine decrease the excretion rate and increase the accumulation of digoxin and digitoxin by HeLa cells. Using cell fractionation we showed that internalized digoxin was retained in the lysosomal compartment of chloroquine treated cells and not on the cell surface. Moreover when cells were grown in the presence of chloroquine they accumulated larger amount of digoxin in the lysosomes. It is believed that the amount of digoxin retained by the cell depends on the lysosomal enzyme activity, once this is inhibited more molecules are accumulated. This is supported by the observed effect of chloroquine on the lysosomal degradation of phospholipids. Chloroquine induced phospholipid accumulation in the

particulate fraction prepared from cells grown in the presence of 10uM chloroquine. This is also an indirect factor which might increase the accumulation of digoxin which has a high lipophilic property, and that this property account for its different behaviour compared with ouabain. The results also show that the ouabain excretion rate has not been affected by chloroquine. It is possible that because ouabain release from its receptor is not pH dependant (Pollack et al., 1981a), and it has poor lipophilic property, could be the reasons behind the lack of effect of chloroquine on ouabain excretion. Thus elevating the intralysosomal pH or inhibiting the lysosomal digestion does not much alter the major step of the excretion of ouabain which is the rate of diffusion out of the cell. Similar observations were obtained when ammonium chloride was used. Moreover, agents which disrupt the tubular structure of the cell such as vinblastine and nocodazole also have no detectable effect on ouabain excretion rate, but digoxin excretion rate was reduced by vinblastine. The previous results do not explain the slight decrease in the accumulation of ouabain in cells grown in the presence of chloroquine which may be due to the decrease of ouabain internalization rate by chloroquine (Griffiths et al., 1983). The possibility of this effect being a result of an increase in the lysosomal enzyme efflux or due to lytic effect induced by chloroquine during the long exposure of

the cell to chloroquine is excluded, as chloroquine showed no such effects on HeLa cells in concentrations as high as 500uM, when both B-hexosaminidase and lactate dehydrogenase activities were detected in the growth medium. Although chloroquine did not affect the cell viability (Griffiths et al., 1983), it greatly decreased B-hexosaminidase specific activity in HeLa cells. This could be a result of the inhibition of the uptake of this enzyme by chloroquine interfering with the delivery of surface bound enzyme to the lysosomes (Sando et al., 1979). Weismann et al., (1975), showed that chloroquine inhibited the uptake and accelerated the efflux of certain acid hydrolases. Chloroquine acceleration of the enzyme efflux has been excluded by measuring the enzyme activity in the cell culture and in the growth medium separately under the same conditions of chloroquine exposure.

Conclusion:-

We think that the process of internalization of cardiac glycosides consist of several steps in which the glycoside is bound to the sodium pump and the pump-ligand complex is internalized and accumulated in a subcellular compartment which appears to be the lysosomes, and then excreted out of the cell. This indicates the importance of internalization of surface bound ligands as a mechanism of cellular clearance of drugs.

The differences in handling by HeLa cells between ouabain and digoxin are consistent with the following model:

1- ouabain, digoxin, and digitoxin all enter the cell in association with the sodium pump.

2- All these cardiac glycosides are then split from the sodium pump on the lysosomal membrane or at prelysosomal vesicles, probably endosomes.

3- ouabain is then retained in the lysosomal interior, perhaps because of its low lipid solubility, and then excreted very slowly from the cell.

4- digoxin and digitoxin are rapidly excreted from the cell, probably because of their high lipid solubility.

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